Vitamin D and Cardiovascular Disease

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VITAMIN D AND CARDIOVASCULAR DISEASE

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A thesis submitted to the University of London for the degree of Doctor of Philosophy in the Faculty of Science

Division of Diabetes and Nutritional Sciences

School of Medicine

King's College London

2014

Publications

Fisk, C.M., Theobald, H.E. & Sanders, T.A.B. (2012). Fortified Malted Milk Drinks Containing Low Dose Ergocalciferol and Cholecalciferol Do Not Differ in Their Capacity to Raise Serum 25-Hydroxy-Vitamin D Concentrations in Healthy Men and Women Not Exposed to UV-B. *J. Nutr*. **142**(7): 1286-1290 (**Appendix 1**)

Fisk, C.M., Theobald, H.E., Chowienczyk, P.J. & Sanders, T.A.B. (2014). Vitamin D and risk of Cardiovascular Disease: a review of the evidence. *Proc. Nutr. Soc.* (Review paper for the 2014 Nutrition Society Summer Meeting Postgraduate Competition, in press). (see **Appendix 2** for abstract submitted for the competition)

Fisk, C.M., Reidlinger, D.P. & Sanders, T.A.B. (2013). Advice to consume 1-2 portions of oily fish per week improves vitamin D status. *Proc. Nutr. Soc.* **72 (OCE4)**, E188 (**Appendix 3**)

Abstract

Evidence for the association between vitamin D status and cardiovascular disease (CVD) is reviewed. Cross-sectional analysis of data from the CRESSIDA and MARINA trials revealed a strong association between vitamin D status and arterial stiffness. An increase in vitamin D status measured as serum 25-OH-D concentrations after following advice to consume 1-2 portions of oily fish/wk was demonstrated (9.2 nmol/L, 95% CI 4.2, 14.2). The effects of malted milk drinks fortified with vitamin D_2 or D_3 at 5 and 10 µg/d *vs.* placebo taken for 4 wk on serum 25-OH-D metabolite concentrations were compared in 8 subjects/group in winter (minimal UVB exposure): mean increments ± SED *vs.* placebo were 9.4 ± 2.5 and 17.8 ± 2.4 nmol/L in 25-OH-D_2 after 5 and 10 µg D_2/d and 15.1 ± 4.7 and 22.9 ± 4.6 nmol/L in 25-OH-D_3 after 5 and 10 µg D_3/d. A total of 41 predominantly normotensive men and post-menopausal women (50-70 y) were randomly allocated to receive 10 µg/d D_2 (Rx) or a placebo malted milk drink for 12 wk in winter. The specified primary outcomes of the trial were 24 h ambulatory blood pressure (BP) and flow mediated dilation (FMD) of the brachial artery. The mean increase ± SED in serum 25-OH-D_2 on Rx *vs.* placebo was 22.8 ± 2.0 nmol/L (P<0.001). The treatment effects (mean changes on Rx *vs*. placebo with 95% CI) were 0.17% (-1.62, 1.28; P=0.82) for FMD and -4.3 mm Hg (-7.3, -1.2; P=0.007) and -2.8 mm Hg (-5.4, -0.2; P=0.032) for systolic and diastolic BP respectively. This BP lowering effect of vitamin D_2 in the winter months and the null finding with regard to FMD need confirmation with a larger sample. A trial of several years duration is required to demonstrate whether the association of PWV with vitamin D status is causal.

Acknowledgements

First and foremost I would like to thank my supervisor Professor Tom Sanders for his constant guidance, inspiration and support and for giving me the freedom to develop into an independent researcher. I would also like to thank Professor Phil Chowienczyk for the guidance and advice he has given me and Dr Hannah Theobald for all her support and encouragement. I am very grateful to all of my supervisors, the Biotechnology and Biological Sciences Research Council, and GlaxoSmithKline for this opportunity which has given me so many new skills and experiences and has been a challenging, but really enjoyable experience.

There are many others whose help has been invaluable. I would like to thank Rosie Calokatsia and Robbie Gray in particular for their technical assistance and constant willingness to provide help and advice, and also Dave Lincoln, Anne-Catherine Perz and MJ Searle. Thank you to Tracy Dew at King's College Hospital for frequently providing me with participant blood test results during screening visits, Crystal Haskell-Ramsay for her help with the cognitive function test and Antony Young for his advice with the UV data.

Many thanks go to Zoe Maniou and Virginia Govoni for being so flexible with their availability for phlebotomy during my busy trials and Ellen Lever and the CRF nurses Dana Navaie, Krzysztof Rutkowski, Ivy Harris, Paula Darroch and Magda Martinez who were a great help with phlebotomy. Thanks also go to Hadiyah Mahmood and Carolyn Major (intercalated medical students) who were always willing to lend a hand wherever help was needed and without whom I couldn't have run my second trial.

Above all, I thank my wonderful husband Jon whose love, patience and encouragement has meant so much. I would also like to thank my parents for their amazing love and support and for always believing in me. Thanks go to my wonderful friends, especially Helen, Izzy, Kristelle and Sarah for being there for me and giving me the motivation to keep going when I needed it! Lastly, my office mates have helped make this experience the enjoyable one that it has been, and thanks go to Androulla, Maryam, Cathriona, Filomena, Fawaz, Sarah, Sowmya, Peiying, Ana, Aseel, Monica,

Oishik, Cathrina, Olah, Nada and Breeana for their friendship, encouragement, constant support and many laughs.

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Abbreviations

$1,25(OH)_2D$	1, 25-dihydroxyvitamin D
$25\text{-}OH\text{-}D_2$	25-hydroxyvitamin D_2
$25\text{-}OH\text{-}D_3$	25-hydroxyvitamin D_3
ABP	Ambulatory blood pressure
ALP	Alkaline phosphatise
Ang 1	Angiotensin 1
AST	Aspartate aminotransferase
ANOVA	Analysis of variance
AUC	Area under the curve
BMI	Body mass index
BP	Blood pressure
CHD	Coronary Heart Disease
CI	Confidence Interval
CIA	Chemiluminescence immunoassay
CPA	Chemical Pathology Accredited
CRESSIDA	Cardiovascular risk Reduction Study: Supported by an Integrated Dietary Approach
CRF	Clinical Research Facility
CRP	C-reactive protein
CV	Coefficient of variation
CVD	Cardiovascular disease
D_2	Ergocalciferol
D_3	Cholecalciferol
DBP	Diastolic blood pressure
DRISK study	The effect of low dose vitamin D_2, provided in a fortified malted milk drink, on cardiovascular risk markers
EIA	Enzyme-linked immunoassay (EIA)
FBC	Full blood count
FFQ	Food frequency questionnaire
FMD	Flow mediated dilatation
$FVII_a$	Activated Factor VII
$FVII_c$	Factor VII coagulant activity
GCE	General Certificate of Education
GGT	Gamma-glutamyl transferase
GM	Geometric mean
GSK	GlaxoSmithKline
HDL-C	High density lipoprotein cholesterol
HOMA-2	Homeostatic model assessment-2
HPLC	High performance liquid chromatography
HND	Higher National Diploma

hsCRF	High sensitivity C-reactive protein
IHD	Ischaemic heart disease
IQR	Interquartile range
KCH	King's College Hospital
KCL	King's College London
LDL-C	Low density lipoprotein cholesterol
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LQ	Lifestyle questionnaire
MARINA study	Modulation of Atherosclerosis Risk by Increasing dose of N-3 fatty Acids
MI	Myocardial infarction
MMP	Matrix metalloproteinase
MRU	Metabolic Research Unit
MUFA	Monounsaturated fatty acids
NO	Nitric Oxide
PI	Principal Investigator
PTH	Parathyroid hormone
PUFA	Polyunsaturated fatty acids
PWV	Pulse wave velocity
REC	Research Ethics Committee
RBC	Red Blood Cells
RIA	Radioimmunoassay
RR	Relative risk
SACN	Scientific Advisory Committee on Nutrition
SBP	Systolic blood pressure
SD	Standard deviation
SFA	Saturated fatty acids
UPLC-MS/MS	Ultra high pressure liquid chromatography tandem mass spectrometry
UVB	Ultraviolet B
VDBP	Vitamin D binding protein
VDR	Vitamin D receptor
WBC	White blood cells
WHO	World Health Organisation

Chapter 1

Introduction

This thesis reviews evidence for the association between vitamin D status and risk of cardiovascular disease (CVD), and examines the effects of vitamin D supplementation on risk factors for CVD and factors associated with the development of CVD. CVD encompasses a number of disorders affecting the heart and vasculature including atherosclerosis, myocardial infarction (MI), peripheral artery disease (PAD), heart failure and stroke/cerebrovascular disease (BHF 2013a). It is the largest cause of death globally and in the UK (WHO 2011; BHF 2013b) with more than a quarter of deaths (> 161,000 people) in the UK occurring as a result of it (BHF 2013b). Coronary heart disease (CHD) is the leading cause of CVD death in the UK, Europe and North America, followed by stroke. However, in many Far Eastern countries, stroke rather than CHD accounts for the highest number of deaths (Gaziano *et al.* 2010). Many factors contribute to risk of CVD notably increasing age, male gender, smoking habit, heredity, exercise and diet. The World Health Organisation defines a risk factor as "any attribute, characteristic or exposure of an individual that increases the likelihood of developing a disease or injury" (WHO 2014). Some factors are unalterable (e.g. age, gender, genetics) but others are modifiable (e.g. smoking habit, high blood pressure (BP), hypercholesterolaemia, hyperglycaemia, obesity). BP is a stronger risk factor for stroke than CHD but elevated serum cholesterol, especially low density lipoprotein (LDL) cholesterol, is the major risk factor for CHD and a weaker risk factor for stroke (Gaziano *et al.* 2010). Various risk algorithms (e.g. QRISK (ClinRisk Ltd 2013), Framingham score) have been developed to estimate CVD risk based on age, gender, BP, lipid profile, smoking habit, body mass index and presence or absence of diabetes). However, whilst these algorithms are useful for identifying high risk individuals for pharmacotherapy they predict less than 45% of CVD events (Hippisley-Cox *et al.* 2008). Thus a large part of the variability in CVD incidence remains unexplained and inclusion of genetic factors does not substantially improve risk prediction (Humphries *et al.* 2010). Novel risk factors may provide prognostic information beyond that of traditional risk factors (Bonetti *et al.* 2003; Cohn 2006). They include high sensitivity CRP (hsCRP), an acute phase reactant, fibrinogen, a plasma glycoprotein involved in clot formation (Torres *et al.* 2003; Kaptoge *et al.* 2012; Danesh *et al.* 2005), and markers of vascular function such as endothelial function and arterial stiffness (Green *et al.* 2011; Yeboah *et al.* 2007; Mattace-Raso *et al.* 2006; Terai *et al.* 2008).

Prospective observational studies have found lower serum 25-hydroxyvitamin D (25-OH-D) concentrations to be associated with a greater risk of CVD mortality, coronary heart disease and stroke (Wang *et al.* 2012b). However, a causal association cannot be inferred because other aspects of lifestyle, such as involvement in physical work outdoors, or disease processes may confound the relationship. Some evidence from long-term randomised controlled trials (RCTs) suggests that vitamin D supplementation (often in combination with calcium) reduces total mortality (Zheng *et al.* 2013; Bjelakovic *et al.* 2014; Chowdhury *et al.* 2014) but there is no clear evidence to support a beneficial effect on CVD incidence and mortality (Bolland *et al.* 2014; Bolland *et al.* 2011; Myung *et al.* 2013).

1.1 Vitamin D

In order to be able to consider the mechanisms behind a potential association between vitamin D and CVD, it is first necessary to understand more about vitamin D. The section that follows provides some background information regarding the history of vitamin D, its chemical forms and sources, absorption, metabolism, physiological role, storage, excretion, biochemical assessment and toxicity as well as a description of status and intake in the UK.

1.1.1 History and discovery

Rickets was documented as a common disease of children in 1650 in England by Francis Glisson (Rajakumar 2003). At the turn of the 20th century, the prevalence of rickets was high in England especially in urban industrial areas. The pall of smoke from coal burning that hung over industrial cities would have decreased exposure to ultraviolet B (UVB) radiation from sunlight and the high consumption of cereals rich in phytate such as oats would have reduced calcium bioavailability (Rajakumar *et al.* 2007). Although using cod liver oil to treat rickets has been recorded as early as 1824, its use for this purpose was questioned and was not widely acknowledged at the time (Rajakumar 2003; Rajakumar *et al.* 2007). However, in 1861, Trousseau correctly deduced that rickets was caused by a lack of exposure to the sun and an inadequate diet, and that cod-liver oil was an effective cure (Rajakumar 2003). In 1890, Palm argued that infants living in Britain were at a greater risk of developing rickets than those living in the tropics even though they had a better diet and improved hygiene.

Later in 1908, the Scottish group of Findlay were able to induce rickets in puppies fed oatmeal and whole milk if they were kept indoors, but reported that puppies given exercise outdoors remained healthy (Findlay 1908). Furthermore, Huldschinsky cured rickets in 1919 in infants using radiation from a sun quartz lamp or carbon arc lamp (Huldschinsky 1928). Subsequent studies showed that exposure of children with rickets to different periods of sunshine in New York City let to marked improvements in the disease (Hess *et al.* 1921).

In 1918, Mellanby, working at King's College for Women in London, showed that cod liver oil cured rickets in puppies (Mellanby 1918). As cod liver oil was also a rich source of vitamin A, some argued that this vitamin might be responsible (Mellanby 1919). However, in 1922, McCollum showed that the anti-rachitic properties remained when the oil was heated to remove its protectiveness against vitamin A deficiency (McCollum *et al.* 1922). He named the anti-rachitic component as 'vitamin D' as only three vitamins had previously been discovered (Rajakumar *et al.* 2007). A trial by Harriet Chick conducted in infants in Vienna at around the same time established beyond doubt that rickets could be prevented by cod liver oil and that the severity of rickets was seasonal (Carpenter 2008). Cod liver oil, in which vitamin D is present as cholecalciferol, or D_3, was then widely administered to children in the UK up until the mid-1950s in order to prevent rickets. However, the Ministry of Health stopped its administration because of concerns around idiopathic hypercalcaemia, a condition that was associated with excess vitamin D that resulted in calcification of soft tissues (Rajakumar *et al.* 2007).

From the 1950s onwards, a large amount of work was conducted to determine the functional metabolism of vitamin D (DeLuca 2014). Vitamin D from dietary and sunlight sources was found to be a precursor for the hormonal metabolite and active form of vitamin D, 1, 25-dihydroxyvitamin D ($1,25(OH)_2D$). $1,25(OH)_2D$ is a steroid hormone as it has a similar molecular structure to classic steroid hormones such as cortisol, and it functions by interacting with a high affinity nuclear hormone receptor, the vitamin D receptor (VDR) which is comparable to other steroid hormones (Norman 2008). Vitamin D is generally only an essential dietary factor in the absence of sunlight exposure, and so is regarded as conditionally essential. However, sunlight exposure is

limited by clothing and is very low in the UK winter months so there is a need to specify a dietary intake for populations who receive little or no sunlight exposure (Vieth 2004a).

1.1.2 Forms

Diet can supply vitamin D_3 (cholecalciferol) and vitamin D_2 (ergocalciferol) (**Figure 1.1**). Vitamin D_3 comprises 27 carbon atoms, and D_2 28 carbons. The difference between them is an additional methyl group in D_2 and a double bond between carbons 22 and 23 (Binkley *et al.* 2011).

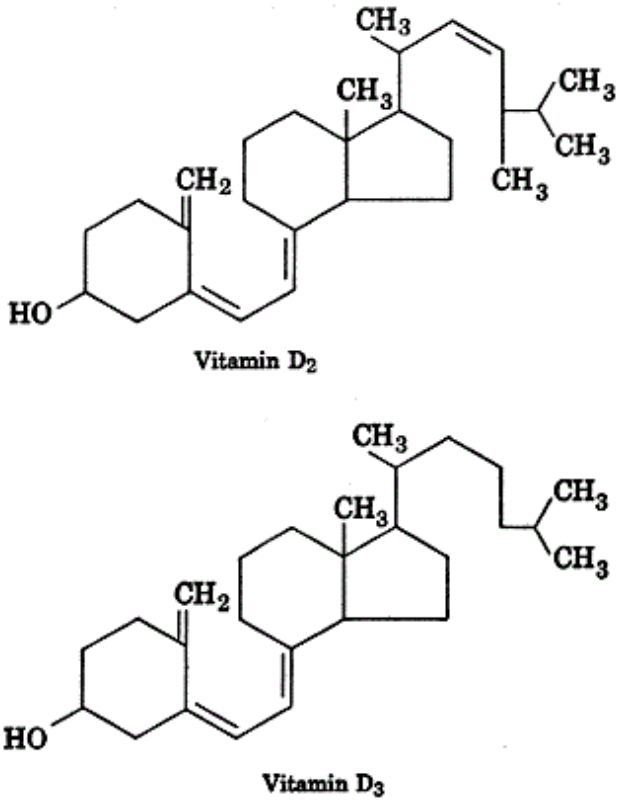


Figure 1.1 Molecular structure of vitamins D_2 and D_3

1.1.2.1 Sources

Most vitamin D is derived from skin exposure to UVB radiation as vitamin D_3. Synthetically D_3 can be produced by irradiating 7-dehydrocholesterol obtained from lanolin in sheep's wool with UVB (Holick 2007). Vitamin D_3 is generally not found in foods of plant origin, although some *Solanum* species contain the $1,25(OH)_2D_3$ glycoside (Wasserman *et al.* 1976). Vitamin D_2 is formed from the action of UVB radiation on ergosterol which is the major sterol in fungi and lower forms of life. Fungi

exposed to sunlight contain significant amounts of vitamin D_2, and D_2 is manufactured commercially by irradiating yeast (Holick *et al.* 2008).

UVB-mediated production of vitamin D begins when 7-dehydrocholesterol in the skin absorbs UVB radiation (290-315 nm) to generate the secosteroid previtamin D_3 (Holick 2007) (**Figure 1.2**). This process also generates steroids including pyrocalciferol, lumisterol and isopyrocalciferol, and the secosteroid tachysterol (Norman 1998). The amount of previtamin D_3 produced for all skin types under normal UVB exposure is not more than about 15% of the available 7-dehydrocholesterol (Webb *et al.* 1988). Any additional sunlight exposure produces the biologically inactive photoproducts lumisterol and tachysterol from the photoisomerisation of previtamin D_3. Following the production of previtamin D_3 in the skin, it is immediately converted to vitamin D_3 in a heat-dependent process via a hydrogen transfer from C-19 to C-9, and vitamin D_3 enters the circulation (Holick 2007).

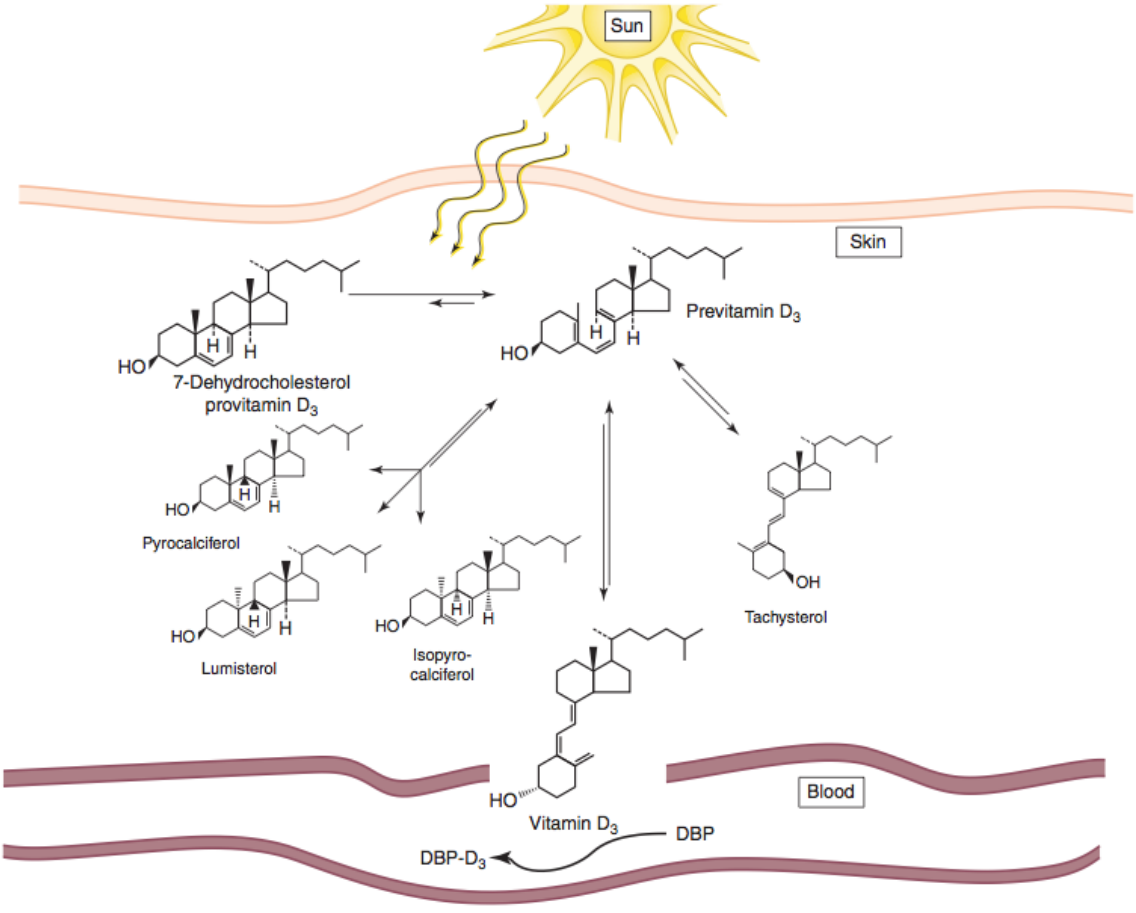


Figure 1.2 Production of vitamin D_3 from 7-dehydrocholesterol taken from (Norman 1998).

There are few sources of vitamin D in the diet. As vitamin D_3, oily fish contains 5-16 µg/100 g, eggs 1.8 µg/100 g and meat ~1 µg/100 g. In the case of poultry the content is greater in the skin at around 1.0 µg/100 g, whereas chicken meat contains less than 0.2 µg/100 g (**Table 1.1**) (McCance and Widdowson 2002). Some fungi contain high amounts of vitamin D_2 (**Table 1.1**). Several foods are fortified with vitamin D notably margarine and yellow fat spreads, some breakfast cereals, yoghurts and infant formulas (Expert Group on Vitamins and Minerals 2003), with most being fortified with D_3 rather than D_2. However, although cow's milk is fortified in the US (10 µg/US quart) and US Airforce bases in the UK, only infant formula has statutory vitamin D fortification in the UK. Margarine has been fortified in the UK by statute (Parliament 1999) to contain not less than 7.05 and not more than 8.82 µg vitamin D per 100 g product, but now legislation is covered by European Community Directives and Regulations. Most yellow fat spreads (except butter), which are not legally classed as margarine because of their lower fat content, are fortified on a voluntary basis at 7.5 µg/100 g end product. Other fortified foods including breakfast cereals and some drinks e.g. (Horlicks™ and Complan™) provide up to about 5 µg/100 g end product (**Table 1.1**). Supplements are a popular source of vitamin D and supplements containing up to 25 µg vitamin D_3 per capsule/tablet can be bought over the counter (**Table 1.1**). Tablets/capsules containing larger amounts >25 µg are pharmacy only products and both D_2 and D_3 are listed in pharmacopoeias as oral forms or prescription products for intra-muscular injections which typically contain 7500 µg to treat or prevent vitamin D deficiency.

Table 1.1 Dietary and supplemental sources of vitamins D_2 and D_3 (McCance and Widdowson 2002; Holick 2006). Adapted from (Holick 2007).

Source	Approximate vit D content (µg)
Foods (average portion size)	
Herring, grilled (100g)	16.1
Kipper, grilled (100 g)	9.4
Mackerel, grilled (100 g)	8.8
Salmon, steamed (100 g)	8.7
Sardines, canned in brine, drained (70 g)	3.2
Tuna, canned in brine, drained (70 g)	2.5
Eggs, chicken, boiled (50 g)	0.9
Liver, lamb, fried (55 g)	0.5
Rump steak, fried, lean (112 g)	0.8
Chicken, roasted, meat, average, light meat (100 g)	0.2
Shiitake mushrooms, fresh (45g)	1.1
Shiitake mushrooms, sun-dried (45g)	17.4
Fortified foods in the UK (average portion size)	
Margarine (10 g)	0.75
Breakfast cereal (30 g)	1.3
Yoghurts (100 g)	0.85
Yoghurt drink (100 g)	0.75
Infant formula (100 mL)	1.2
Soya milk (250 mL)	1.9
Supplements available over the counter	
Multivitamin	5-10 D_2 or D_3
Vitamin D_3	10 or 25 D_3

1.1.2.2 Comparison of vitamin D_2 and D_3

In the 1930s, vitamin D_2 and D_3 were found to be effective in preventing and treating infantile rickets (Trang *et al.* 1998). It was concluded in 1940 that "For practical purposes, the vitamin D in vitosterol (vitamin D_2) may be regarded as being equal to the vitamin D in cod liver oil (vitamin D_3)" (Park 1940) and pharmacopoeias still regard the two forms as equivalent and interchangeable with 1 international unit (IU) being equivalent to 25 ng of D_2 or D_3, even though D_3 has a marginally lower molecular weight (384 g/mol) compared with D_2 (399 g/mol) (Medicines Commission 1980; Committee of Revision 1997). Furthermore, studies have shown the two forms to be equally effective in treating hypovitaminosis D in healthy and rachitic infants and

children (Gordon *et al.* 2008; Thacher *et al.* 2010). Holick (Holick *et al.* 2008), a leading authority on vitamin D, continues to maintain that both forms are equipotent. Houghton and Vieth on the other hand suggest that D_2 is inferior to D_3 (Houghton *et al.* 2006). In support of this, a meta-analysis of 7 RCTs which gave vitamin D as a bolus or daily dose, compared the ability of the two forms to increase serum concentrations of 25-hydroxyvitamin D (25-OH-D), the major circulating form of vitamin D, and concluded that D_2 was less effective than D_3 (weighted mean difference of 15.23 nmol/L, 95% CI 6.12, 24.34) (Tripkovic *et al.* 2012). However, the comparison for daily vitamin D supplementation found no significant difference between D_2 and D_3 (weighted mean difference 4.83 nmol/L, 95% CI -0.98, 10.64). Furthermore, the conclusions of these meta-analyses are questionable as some of the studies included did not adjust for sunlight exposure (Binkley *et al.* 2011; Armas *et al.* 2004) or did not use assays that were specific for the two different 25-OH-D metabolites, 25-OH-D_2 and 25-OH-D_3 (Romagnoli *et al.* 2008; Trang *et al.* 1998; Heaney *et al.* 2011; Armas *et al.* 2004). Some studies also gave pharmacological doses of vitamin D by non-oral routes (ie. intramuscular administration of 7500 μg (Leventis *et al.* 2009)) or in tablet or capsule form (i.e. a single dose of 7500 μg (Romagnoli *et al.* 2008) or weekly dose of 1250 μg (Heaney *et al.* 2011) and so are not appropriate comparisons for dietary intake where recommended intakes are 10 μg/d (Scientific Advisory Committee on Nutrition 2007).

In relation to the biological activity of the two forms of vitamin D, some studies have shown 25-OH-D_2 to have a lower affinity for the vitamin D binding protein (VDBP) compared with 25-OH-D_3 (Hollis 1984; Nilsson *et al.* 1972). It has been suggested that this would lead to 25-OH-D_2 having a shorter circulating half-life and a greater clearance rate. However, it would also result in greater free, unbound concentrations of 25-OH-D_2. It has been suggested that the 25-hydroxylase enzyme may have a lower affinity for vitamin D_2 than vitamin D_3 resulting in a greater rate of production of 25-OH-D_3 compared with 25-OH-D_2. A study in subcellular fractions of human liver found a five times greater rate of mitochondrial hydroxylation of D_3 compared with D_2 (Holmberg *et al.* 1986), although these results are questionable as the assay used to detect 25-OH-D_2 was less sensitive than that for 25-OH-D_3, and another study in chick liver tissues found no difference in the rate at which 25-OH-D_2 and 25-OH-D_3 were

formed (Jones *et al.* 1976). After formation of the active form of vitamin D from both D_2 and D_3, a 24-hydroxylation step occurs converting $1,25(OH)_2D$ to $1,24,25(OH)_3D$. The formation of $1,24,25(OH)_3D_2$ deactivates vitamin D_2, but $1,24,25(OH)_3D_3$ continues to be biologically active and is still able to bind to the vitamin D receptor (VDR) as a further side-chain oxidation is needed to deactivate it (Horst *et al.* 1986; Houghton and Vieth 2006). However, the above differences in metabolism between the two forms do not necessarily mean that biological activity will differ. Many of these findings are based on experiments in animals, and it needs to be recognised that there are large species differences in the metabolism of vitamin D which limits the translation of findings in animals to man. In chicks, for example, vitamin D_2 only has 8-11% of the activity of D_3 in preventing rickets (Chen *et al.* 1964). Data on the comparative toxicity of vitamin D_2 and D_3 in humans are lacking according to the Expert Group on Vitamins and Minerals. However, vitamin D_3 is significantly more toxic than D_2 in non-human primates (Rhesus monkey) (Expert Group on Vitamins and Minerals 2003).

1.1.3 Absorption, metabolism, storage and excretion

Vitamin D is a steroid with chemical properties similar to cholesterol, being better absorbed dissolved in fat or in the presence of fat in the small intestine (Haddad *et al.* 1993; IOM 2011). It is absorbed in lipid-containing micelles which diffuse into enterocytes (IOM 2011). This is likely to be why studies have shown absorption to be more efficient if vitamin D is consumed with fat-containing foods (Weber 1981). Both vitamin D_2 and D_3 from food and supplements, together with other sterols, are incorporated into chylomicrons in the intestinal wall and rapidly transported by the lymphatic system into the circulation (Holick 2007).

Vitamin D in the circulation from sunlight and dietary sources is bound to the VDBP and transported to the liver (Holick 2007; Norman 1998). In the liver, 25-OH-D is formed from the addition of a hydroxyl group to the 25 position of the vitamin D molecule by 25-hydroxylase (**Figure 1.3**). 25-OH-D is biologically inactive and the major circulating form of vitamin D and is used to determine vitamin D status. It is transported in the circulation to the kidneys where it is converted to the active form $1,25(OH)_2D$ by the enzyme 25-hydroxyvitamin D-1α-hydroxylase (1α-hydroxylase) (Holick 2007). When serum calcium levels are low, parathyroid hormone (PTH) is

released which stimulates a greater production of 1,25$(OH)_2$D. This has the effect of increasing serum calcium concentrations by increasing bone calcium re-absorption, increasing the intestinal absorption of calcium and phosphate, and decreasing the renal excretion of calcium (Makariou *et al.* 2011). A negative feedback loop then leads to a decreased synthesis of 1,25$(OH)_2$D, and a decreased synthesis and secretion of PTH (Holick 2007). Levels of 1,25$(OH)_2$D are tightly regulated in the blood and only lowered when there is a severe deficiency of 25-OH-D.

Storage of vitamin D in the body is mainly as 25-OH-D bound to VDBP in the circulation, but it can also be stored in the liver or fat cells and later released (Holick 2007). Experimental studies in rats have shown adipose tissue to accumulate the most vitamin D and have the slowest rate of release (Rosenstreich *et al.* 1971). Furthermore, serum 25-OH-D concentrations have been observed to increase after weight loss in obese subjects (Tzotzas *et al.* 2010).

Calcitroic acid, a water soluble and biologically inactive form of vitamin D is the main excretory product of vitamin D which is excreted in the bile in the faeces (**Figure 1.3**). It is formed from the catabolism of 1,25$(OH)_2$D and 25-OH-D by the enzyme 25-hydroxyvitamin D-24-hydroxylase (CYP24) (Makariou *et al.* 2011; Holick 2007). Very little vitamin D is excreted in urine (IOM 2011).

An alternative metabolic pathway through which vitamin D_3 can be metabolised is the C-3 epimerisation pathway. This involves the conversion of the hydroxyl group at position C-3 of the A-ring from the alpha to the beta orientation, resulting in stereoisomers of vitamin D metabolites (Bailey *et al*. 2013). Evidence from *in vitro* studies supports that the epimers 3-epi-25-OH-D_3, 3-epi-1α,25$(OH)_2D_3$ and 3-epi-24,25$(OH)_2D_3$ can be produced via this pathway (Kamao *et al*. 2004) and the 3-epi-25-OH-D_3 has been identified in infants, children and adults (van der Ouweland *et al*. 2011). The epimerisation appears to take place in extrarenal tissues, but it is not yet known which enzymes are responsible (Bailey *et al*. 2013). Currently the biological function of the epimers is uncertain, although evidence suggests that 3-epi-1α,25$(OH)_2D_3$ may possess some but not all of the regulatory effects of 1,25$(OH)_2$D (Bailey *et al*. 2013; Kamao *et al*. 2004). PTH secretion has been shown to be

suppressed to a similar extent by both forms (Brown *et al*. 1999), but 3-epi-1α,25(OH)$_2$D$_3$ had only 12-13% of the transcriptional activity of 1,25(OH)$_2$D for inducing the expression of the human osteocalcin and CYP24 genes (Kamao *et al*. 2004).

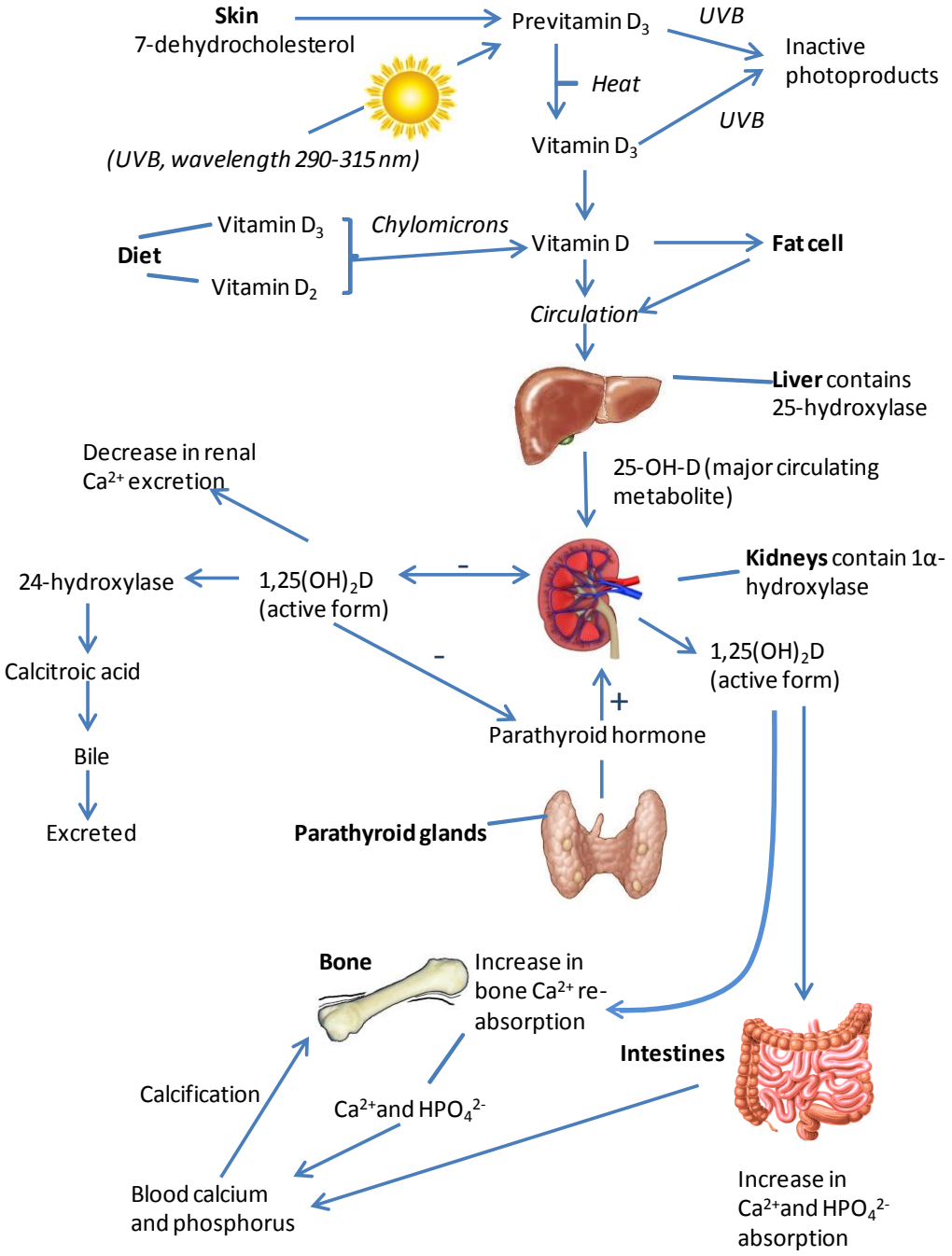


Figure 1.3 Metabolism of vitamin D in the body adapted from (Holick 2007). HPO_4^{2-}, hydrogen phosphate; Ca^{2+}, calcium; 25-OH-D, 25-hydroxyvitamin D; 1,25(OH)$_2$D, 1, 25-dihydroxyvitamin D; UVB, ultraviolet B radiation.

1.1.4 Biochemical assessment of vitamin D status

There are many different vitamin D metabolites, but there is some uncertainty as to which form best represents status and the bioavailability of vitamin D. Currently, although $1,25(OH)_2D$ is the active form, vitamin D status is measured as plasma or serum concentrations of 25-OH-D. $1,25(OH)_2D$ is present in the blood at very low concentrations and is unsuitable as a measure of status as it can be at normal or elevated levels even when a patient is vitamin D insufficient (Holick 2009). Furthermore, the half-life of $1,25(OH)_2D$ in the circulation is only 4-6 h (Holick 2009), compared to about 2-3 wk for 25-OH-D (Lund *et al.* 1980). Serum/plasma 25-OH-D is not under tight homeostatic regulation and is therefore useful as it is representative of the supply and usage of vitamin D over a time period (Prentice *et al.* 2008). Its concentrations are dependent on the amount of vitamin D reaching the liver from dietary and sunlight sources, how much 25-OH-D the liver produces and how long it lasts in the circulation (its half-life). However, total 25-OH-D is not necessarily a good indicator of the ability of vitamin D to meet the body's functional requirements (Prentice *et al.* 2008). Free or bioavailable 25-OH-D may provide a more meaningful marker of vitamin D status (Chun *et al.* 2013; Glendenning *et al.* 2013). Less than 1% of 25-OH-D in the circulation exists as free and unbound; about 85-90% is bound with high affinity to DBP and a further 10-15% is bound to albumin (Bikle *et al.* 1986). Some authors have labelled the albumin-bound and free form together as 'bioavailable' 25-OH-D because when it is bound to the VDBP some of its actions may be inhibited, preventing it from acting on target cells, and it binds to albumin with a lower affinity than VDBP (Bikle *et al.* 1989; Safadi *et al.* 1999). Powe *et al.* found that black Americans had lower total 25-OH-D concentrations compared to whites, but because they also had lower concentrations of VDBP, the amount of bioavailable 25-OH-D for both ethnic groups was similar (Powe *et al.* 2013). The same group found free 25-OH-D concentrations to be better correlated with bone mineral density than total 25-OH-D concentrations in young adults (Powe *et al.* 2011). It is hypothesised therefore that only free and bioavailable 25-OH-D are available for cellular processes making them a better indicator of physiological bioactivity compared to total 25-OH-D (Glendenning *et al.* 2013; Powe *et al.* 2011; Al-oanzi *et al.* 2006).

In terms of representing function, PTH has also been suggested as a useful biomarker of vitamin D (Prentice *et al.* 2008). It is well-established that strong inverse correlations exist between plasma PTH and 25-OH-D concentrations (Bates *et al.* 2003; Krall *et al.* 1989; Chapuy *et al.* 1997), although some suggest that PTH no longer falls when serum 25-OH-D reaches a certain level (Need *et al.* 2000; Chapuy *et al.* 1997; Lappe *et al.* 2006). However, it is important to note that 25-OH-D concentrations vary considerably at a given concentration of PTH and that PTH is not specific for vitamin D deficiency (Lips *et al.* 1988; Dawson-Hughes *et al.* 1997). Furthermore, the interaction between PTH and 25-OH-D may vary in different population groups as, for example, elevated PTH concentrations have been found in Africans who have a good vitamin D status, but a low calcium intake (Aspray *et al.* 2005).

In summary, 25-OH-D is the preferred marker of vitamin D status, but it may also be useful to measure free and bioavailable 25-OH-D.

1.1.5 Definition of vitamin D insufficiency and prevalence in the UK

In the early 1970s adequate serum 25-OH-D concentrations used to be defined based on the levels that would be required to prevent osteomalacia or rickets. More recently, definitions have been based on the concentration that maximally suppresses PTH. However, estimates vary, and if vitamin D concentrations are causally associated with health outcomes other than bone health, defining an optimum intake is more complex. A review has concluded that the most advantageous serum concentrations of 25-OH-D begin at 75 nmol/L after summarising the evidence from previous studies that had evaluated the threshold for serum 25-OH-D concentrations in relation to bone mineral density (BMD), lower extremity function, dental health, risk of falls and fractures, and colorectal function (Bischoff-Ferrari *et al.* 2006). Whilst in agreement with this the Endocrine Society define serum 25-OH-D concentrations of 52.5-72.5 nmol/L as insufficient, most public health organisations have advised that 50 nmol/L should be sufficient to meet the needs of most people (**Table 1.2**). Vitamin D deficiency is usually defined as serum 25-OH-D concentrations less than 25 or 30 nmol/L (**Table 1.2**) and this may, although not in all cases, lead to rickets in children. In adults vitamin D deficiency causes a decrease in the efficiency of calcium and phosphorus absorption from the intestines and can result in secondary hyperparathyroidism which causes a

decrease in bone mineral density and the development of osteomalacia. This results in muscle and bone weakness and an increased risk of fractures in the elderly due to more frequent falls (Expert Group on Vitamins and Minerals 2003; Holick 2006; Holick 2007; Zittermann *et al.* 2005).

Table 1.2 Serum 25-OH-D thresholds used to assess vitamin D deficiency and sufficiency by different public health organisations.

Public health organisation	Serum 25-OH-D thresholds
Institute of Medicine [1]	25-OH-D at 50 nmol/L should cover the needs of 97.5% of the population. 40 nmol/L is the median requirement. Deficiency symptoms may appear <30 nmol/L depending on a range of factors.
The Endocrine Society [2]	Deficiency is serum 25-OH-D <50 nmol/L and insufficiency 50-72.5 nmol/L.
National Osteoporosis Society [3]	Deficiency is serum 25-OH-D <30 nmol/L. Concentrations 30-50 nmol/L may be inadequate in some people and 25-OH-D>50 nmol/L is sufficient for almost the whole population.
Scientific Advisory Committee on Nutrition [4]	A Vitamin D Working Group has been established to consider thresholds used and it has been agreed that achieving a serum 25-OH-D concentration of at least 25 nmol/L should be used as the criterion for establishing the Reference Nutrient Intake (RNI) for vitamin D.

1 (IOM 2011); 2 (Holick *et al*. 2011); 3 (National Osteoporosis Society 2013); 4 (SACN 2014).

Vitamin D insufficiency is common in the UK (Bates *et al.* 2014; Hypponen *et al.* 2007), particularly in the winter when a marked seasonal fall in plasma 25-OH-D concentrations occurs with the nadir in February/March (**Figure 1.4**). The fall is attributed to minimal UVB exposure between November and March due to the UK's location between 50 ˚and 60 ˚N. Around 55-75% of the population exhibits concentrations below 50 nmol/L in the winter, and 17-39% have values below 25 nmol/L (Bates *et al.* 2014; Ruston 2004). These proportions are lower in the summer months (**Figure 1.4**). Data from the most recent 2008-2012 National Diet and Nutrition Survey (NDNS) of 1769 individuals found mean 25-OH-D concentrations of 43.5 nmol/l in men and 47.3 nmol/l in women aged 19-64 y from blood samples collected throughout the year (Bates *et al.* 2014). Compared to these, concentrations were higher in men (47.3 nmol/L) and lower in women (42.5 nmol/L) in the older age group of 65+ y (Bates *et al.* 2014). Large differences in status between ethnicities have also

been found; in a study of 500 pregnant adolescents in the UK, serum 25-OH-D concentrations were significantly higher in white and mixed white-Caribbean subjects compared to black African and Caribbean subjects (P<0.001) and values were only seasonal in the whites and mixed-white Caribbean subjects (Baker *et al.* 2009). Additionally, in 830 outpatients from a multi-ethnic population in Birmingham, vitamin D deficiency defined as serum 25-OH-D <25 nmol/L was much more prevalent in black Afro-Caribbeans (31%) and Asians (36%) compared to whites (12%) (Ford *et al.* 2006).

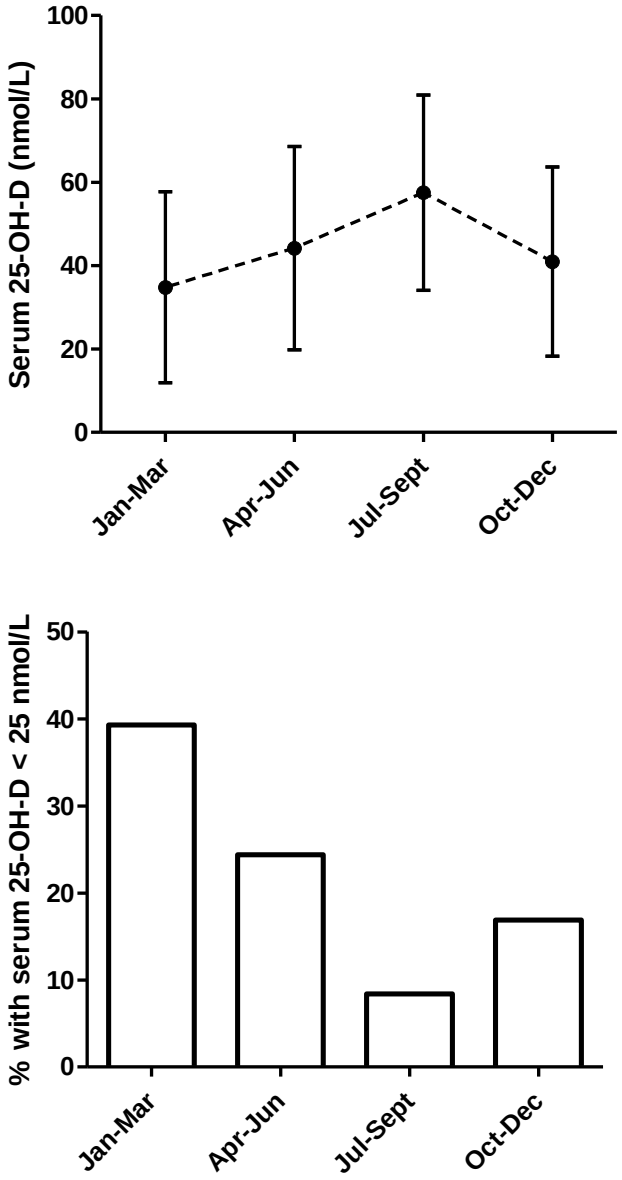


Figure 1.4 Mean (SD) serum 25-OH-D concentrations measured throughout the year in 19-64 y olds in the 2008-2012 National Diet and Nutrition Survey (NDNS) (top) and percentage of participants with serum 25-OH-D<25 nmol/L (bottom) (Bates *et al.* 2014).

1.1.6 Factors influencing vitamin D status

Certain groups of people including older institutionalised individuals who are confined indoors and therefore do not have sufficient UVB exposure and the obese are at a greater risk of vitamin D deficiency compared to others. Obese subjects have been shown to have lower 25-OH-D concentrations than normal weight controls, and inverse associations have been found between BMI and serum 25-OH-D (Wortsman *et al.* 2000). It is thought that vitamin D is deposited in body fat compartments leading to a reduced bioavailability (Wortsman *et al.* 2000). Chronic renal or liver disease and inflammatory bowel disease patients are also at an increased risk of deficiency as disease processes result in a decreased capacity for intestinal absorption of vitamin D (Expert Group on Vitamins and Minerals 2003). Furthermore, certain medications, including anticonvulsants which, for example, are used to treat HIV/AIDS, can lead to a lower vitamin D status as they enhance the catabolism of 25-OH-D and $1,25(OH)_2D$ (Zhou *et al.* 2006). High intakes of unrefined cereals may also increase the catabolism of vitamin D, most likely due to their high phytic acid content interfering with calcium absorption (Dagnelie *et al.* 1990). It has been found in twin studies that there is a large genetic contribution of around 40% to 25-OH-D concentrations (Hunter *et al.* 2001), and this may be due to genetic polymorphisms in the VDBP. Other studies have shown these polymorphisms to be responsible for a greater proportion of the variability in serum 25-OH-D than other factors including age and BMI (Powe *et al.* 2013), and healthy individuals have been found to have different percentage increases in 25-OH-D concentrations in response to vitamin D supplementation dependent on their VDBP genotype (Fu *et al.* 2009).

Certain factors influence the ability of the skin to produce vitamin D including aging, use of a sunscreen or clothing/headscarves which cover the skin, and increased skin pigmentation (Holick *et al.* 2011). Aging has been shown to be associated with decreased concentrations of 7-dehydrocholesterol in the skin (MacLaughlin *et al.* 1985), and older people may be less likely to spend time outdoors. Sunscreen use, even at a low sun protection factor of 15 can block up to 99% of vitamin D synthesis in the skin (Pearce and Cheetham 2010). Skin synthesis is also blocked through the wearing of a veil or concealing clothing which prevents UVB radiation reaching the skin (Pearce and Cheetham 2010). Dark skin leads to a reduced capacity of the body to

produce vitamin D as melanin skin pigmentation competes for and absorbs UVB photons that are responsible for the photolysis of 7-dehydrocholesterol to previtamin D. This means that a person with dark skin may need to spend around 3-5 times as long in the sun compared to a person with white skin in order to make an equivalent amount of vitamin D (Clemens *et al.* 1982). Immigrants with darker skin moving to northern latitudes are particularly at risk of developing vitamin D deficiency (Solanki *et al.* 1995).

Environmental factors including latitude, altitude, season and time of day have a huge influence on 25-OH-D concentrations as they affect the zenith angle of the sun and therefore the amount of UVB radiation that people are exposed to. The level of UVB radiation is lower at lower altitudes and at higher latitudes (Webb *et al.* 1988; Engelsen *et al.* 2005); above a latitude of 37 ˚ in the winter months there is very little, if any, vitamin D_3 synthesis in the skin (Holick 2004). In the UK, a gradient in the prevalence of vitamin D deficiency occurs with it decreasing from Scotland to the South of England (Hypponen and Power 2007).

1.1.7 Toxicity

Whilst vitamin D deficiency is a health concern, it is also important to consider whether there are any negative consequences of too much vitamin D, particularly in relation to widespread food fortification where it is difficult to decide on a level suitable to meet the needs of the population at the same time as trying to ensure there are no toxic effects. In terms of vitamin D production from excessive UVB exposure, toxicity does not occur as any excess previtamin D_3 or vitamin D_3 is destroyed by sunlight (Holick 2007). This occurs because the photolysis of 7-dehydrocholesterol is reversible and previtamin D is converted to the biologically inactive tachysterol by photo-isomerisation (Norval *et al.* 2010). However, in relation to exogenous sources of vitamin D, there is evidence that very high levels of supplementation are a cause of hypercalcaemia and hypercalciuria (Johnson *et al.* 1980; Jacobus *et al.* 1992). The physiological role of vitamin D is primarily to promote calcium absorption and bone resorption (see section 1.1.3). In excess, vitamin D results in the calcification of soft tissues (Expert Group on Vitamins and Minerals 2003) including the vasculature and causes renal damage (McConnell *et al.* 2009). The

toxicity of vitamin D is well known to veterinarians in a variety of species. At one stage vitamin D_3 was used as a rodenticide because of its high toxicity per kg body weight (Murphy 2002). The mechanisms as to how hypervitaminosis D leads to toxicity are unclear (Jones 2008) although a few hypotheses have been put forward, with the most likely explanation being that the excess 25-OH-D causes displacement of $1,25(OH)_2D$ from the VDBP resulting in excessive levels of "free" $1,25(OH)_2D$ which may cause inappropriate gene expression (Vieth 1990; Jones 2008). Over-fortification of milk with vitamin D in a population of 11,000 households in Boston between 1987 and 1991 is thought to have led to 56 cases of hypervitaminosis D, defined as serum 25-OH-D ≥225 nmol/L, and 2 deaths related to hypercalcaemia (Blank *et al.* 1995). The dairy supplying the milk to the community was adding an unmeasured amount of vitamin D to the milk due to a broken instrument. Mandatory fortification of milk required that no more than 12.5 μg vitamin D was added per quart, but analysis of milk samples from the dairy indicated levels of between 70 and 600 times this. Strong correlations were observed between hypervitaminosis D and consumption of milk from this particular dairy, implying that the milk was the cause of the hypervitaminosis D. Symptoms of these individuals included anorexia, weakness, fatigue, weight loss and dehydration, and at least 41 were hospitalised (Blank *et al.* 1995). In a trial by Johnson *et al.* in which 63 individuals (females over 60 y, males over 65 y) took vials of oil containing 50 μg vitamin D/d for 6 months, two individuals developed hypercalcaemia (Johnson *et al.* 1980). However, other studies in which 100 μg/d was given to 61 healthy men and women aged 41 ± 9 y for 2-5 months (Vieth *et al.* 2001), or 32 male and female outpatients aged 55 ± 9 for more than 6 months were unable to detect a change in serum or urine calcium (Vieth *et al.* 2004b).

Due to a lack of data, the UK has not established a safe upper level for long-term vitamin D intake, although for guidance purposes, 25 μg/d has been considered as safe and unlikely to cause adverse effects in the general population (Bassuk *et al.* 2009). In North America and Europe the tolerable upper intake level is 50 μg/d at which no adverse health effects are likely (European Commission Health Consumer Protection Directorate-General 2002; IOM 1997); for a 50 kg reference woman that would be 1 μg/kg. However, a review by Vieth in 2007 claims that 50 μg/d is too conservative and that long-term intakes of up to 250 μg/d of D_3 are unlikely to cause adverse effects in

the majority of the general population (Vieth 2007b). There appears to be very little evidence for this conclusion though. In summary, whilst it would seem from the above that it is better to source vitamin D from sunlight as it poses no risks of toxicity, concerns over the risk of skin cancer, and difficulties in obtaining sufficient UVB exposure in certain countries and particular groups of people (see section 1.2.5) mean that diet remains an important source.

1.1.8 Intake in the UK

In the UK, the most recent dietary reference values were set in 1991 (Committee on Medical Aspects of Food Policy 1991). They were based on the vitamin D intake required to maintain 25-OH-D concentrations during the winter months and prevent deficiency (Committee on Medical Aspects of Food Policy 1991). Pregnant and lactating women, adults over 65 y of age, and specific at-risk groups who are not receiving sufficient UVB exposure due to, for example, being confined indoors or wearing clothing that fully covers the skin, have a Reference Nutrient Intake (RNI) of 10 µg/d (Scientific Advisory Committee on Nutrition 2007). Infants 0 to 6 months and 7 months to 3 y have a RNI of 8.5 and 7 µg/d, respectively. Children and adults aged 4-64 y were assumed to obtain sufficient vitamin D from UVB exposure and therefore there are currently no RNIs for people in this age group. In 1998, a review was conducted by the Department of Health to assess evidence on the association between 25-OH-D concentrations and bone health. The Committee, based on the conclusions of the review, decided that no changes to the RNIs were required (Department of Health 1998). More recently, a Scientific Advisory Committee on Nutrition (SACN) Working Group was assembled in 2011 to "review the Dietary Reference Values for vitamin D intake and make recommendations" (Scientific Advisory Committee on Nutrition 2014). Although there is no current UK RNI for adults, for food labelling purposes, an intake of 5 µg represents the recommended daily amount in the European Union (European Commission Health Consumer Protection Directorate-General 2008). Vitamin D intake recommendations in other parts of the world are similar to those of the UK and most other countries have published recommendations for adults as well (**Table 1.3**).

Table 1.3 Recommended vitamin D intakes (µg/d) in different countries

	Infants<1y	Young children 1-3 y	Children 4-10 y	Adolescents 11-18 y	Adults	Older adults
IOM (US) [1]	10	15	15	15	15	20
Ireland [2]	7.5-8.5	10	0-10	0-15	0-10	10
Netherlands [3]	10	10	10	10	10	20
Nordic countries [4]	10	10	10	10	10	20
UK [5]	8.5	7	-	-	-	10
WHO/FAO [6]	5	5	5	5	5	10-15

IOM, Institute of Medicine (US); WHO/FAO, World Health Organisation/Food and Agriculture Organization for the United Nations. 1 (IOM 2011); 2 (Food Safety Authority of Ireland 1999); 3 (Health Council of the Netherlands 2012); 4 (Nordic Council of Ministers 2012); 5 (Committee on Medical Aspects of Food Policy 1991); 6 (FAO 2001).

The most recent UK nationwide assessment of vitamin D intake was the NDNS Rolling Programme for which data has been published on information collected between 2008 and 2012 (Bates *et al.* 2014). For adults aged over 65 y (*n*=753), the mean dietary vitamin D intake from food sources was only 33% of the RNI of 10 µg/d, although inclusion of supplements increased this to 51% (Bates *et al.* 2014). In children aged 1.5-3 y of age (*n*=604) for whom the RNI is 7 µg/d, the corresponding intakes were 27% and 32% of the RNI. For adults aged 19-64 (*n*=2697), the median vitamin D intakes from foods sources only were 2.5 µg/d in men (*n*=1126) and 2.1 µg/d (*n*=1571) in women (Bates *et al.* 2014). When supplements were included, intakes increased to 2.7 µg/d in the men and 2.5 µg/d in the women. The intakes were higher in men and women aged 65 y and over. From food sources only they were 3.2 and 2.5 µg/d respectively, but they increased to 5.1 and 5.2 µg/d respectively on inclusion of supplements.

In adults aged 19-64, vitamin D in the diet was coming from meat and meat products (30%), fat spreads (19%), fish and fish dishes (17%), cereals and cereal products (13%), eggs and egg dishes (13%), and milk and milk products (5%). Most (65%) of the vitamin D from the fish and fish dishes came from oily fish (Bates *et al.* 2014). Another survey of 1379 adults aged 18-64 y from the North/South Ireland Food Consumption Survey

found similar results to the NDNS; vitamin D intake from food sources only was 3.7 μg/d in men and 2.8 μg/d in women, and this increased to 4.4 μg/d and 4.0 μg/d when supplements were included. Meat and meat products were the primary contributors to vitamin D intake (30.1%), although fish and fish products (14.3%) and eggs and egg dishes (9.1%) were also important. Compared to the above findings, a study in 2235 healthy adults aged 21-82 y from Scotland found slightly higher vitamin D intakes. The mean intake from food sources was 4.7 μg/d in the 74% of participants who did not take supplements. When looking at intake from food sources and supplements, the mean intakes were 9.8 μg/d in men and 8.1 μg/d in women. Here the most important dietary contribution to vitamin D intake was fish, followed by meats, eggs, cakes and breakfast cereals (Zgaga *et al.* 2011). Although the contribution from cakes may be partly due to the mandatory fortification of margarine, the contribution of fortified foods to dietary vitamin D intake is very small compared to in the US where it is the main contributor, mostly as fortified milk (Moore *et al.* 2005).

Whilst research suggests that UVB exposure contributes more to average vitamin D status than diet (Ashwell *et al.* 2010), the above figures from the different surveys show that adults in the UK are not receiving enough vitamin D from the diet based on the recommendation of 10 μg/d for those confined indoors or the even higher 15 μg RDA in the US (IOM 2011). However, an important question is if dietary intake was averaging at 10 μg/d or more, would this actually be enough to produce an adequate vitamin D status in the majority of the general population, particularly in the winter months when there is very little UVB exposure? In terms of preventing deficiency, 2 Irish RCTs (Cashman *et al.* 2008; Cashman *et al.* 2009) which gave 238 adults aged 20-40 y and 225 adults aged 64+ y doses of 0, 5, 10 or 15 μg/d vitamin D_3 for 22 wk in the winter months found that an intake of 9 μg vitamin D/d would be needed to maintain winter 25-OH-D concentrations in 97.5% of the population above 25 nmol/L. However, to maintain levels at more than 50 nmol/L in 97.5% of the population, it was estimated that a much higher intake of 28 μg/d would be needed for adults aged 20-40 y and 25 μg/d for adults aged 64+ y (Cashman *et al.* 2008; Cashman *et al.* 2009).

The fact that dietary requirements for vitamin D vary markedly between different groups of people and also within individuals at different times of the year due to

variations in UVB exposure means that it is difficult to define recommended intakes. Furthermore, the lack of availability of vitamin D in foods makes it difficult to obtain even low intakes from the diet, although food fortification and supplementation help boost intakes.

1.1.9 Actions and effects of vitamin D

1.1.9.1 Calciotropic

The calciotropic actions of vitamin D, as described in section 1.1.3, are fairly well established. In its active form, vitamin D causes a number of effects in order to maintain adequate concentrations of calcium (Ca^{2+}) and phosphorus (HPO_4^{2-}) in the blood to promote mineralisation of the skeleton (McConnell *et al.* 2009). Initially when plasma calcium levels drop in the body, PTH is released leading to the conversion of 25-OH-D to 1,25$(OH)_2$D which then interacts with the vitamin D receptor-retinoic acid x-receptor complex (VDR-RXR). This leads to an increased expression of the epithelial calcium channel and calbindin, a calcium binding protein, resulting in an increased intestinal absorption of calcium (Holick 2007). An effect on bone resorption occurs when the VDR present in osteoblasts (bone-forming cells) recognises 1,25$(OH)_2$D causing the cells to increase their expression of the receptor activator of nuclear factor-κB ligand (RANKL). RANKL binds to its receptor RANK on preosteoclasts which are then converted to mature osteoclasts (bone cells that resorb bone tissue) (Holick 2007). The increase in PTH also enhances renal reabsorption of calcium and decreases phosphorus reabsorption (Holick 2006). In vitamin D deficiency, the absorption of calcium and phosphorus from the diet can be reduced to levels of only 10-15% and 50-60%, respectively, and this can cause rickets in children and osteomalacia in adults (Holick 2006). Usually infants and children with rickets have serum 25-OH-D concentration <25 nmol/L (Holick 2006). However, this is not always the case as African and Bangladeshi children with rickets have been shown to have no evidence of vitamin D deficiency (Pfitzner *et al.* 1998; Fischer *et al.* 1999). It is thought that rickets is occurring in these children due to a low calcium supply which leads to urinary phosphate wasting (Braithwaite *et al.* 2012).

1.1.9.2 Non-calciotropic

Evidence has emerged to suggest potential effects of vitamin D on other aspects of health. Studies have shown associations between vitamin D status and cancer, autoimmune disease, cardiovascular disease (CVD), infectious diseases, obesity and even cognitive function (Wang *et al.* 2012b; Bolland *et al.* 2014; Myhr 2009; Annweiler *et al.* 2013; Nnoaham *et al.* 2008). There are also studies to suggest that $1,25(OH)_2D$ stimulates insulin secretion in β cells in the pancreas (Gedik *et al.* 1986; Kadowaki *et al.* 1984). This area of research is expanding and developing but it is unknown as to whether vitamin D deficiency is a consequence of, or plays a causal role in disease processes. Much of the research stems from the discovery over the last few decades of the expression of the VDR and 1α-hydroxylase in different tissue types (Zehnder *et al.* 2002; Hewison *et al.* 2007; Rahman *et al.* 2007; Wang *et al.* 2008; Giovannucci 2009). The active form of vitamin D, $1,25(OH)_2D$, produced by the action of 1α-hydroxylase on 25-OH-D, exerts its effects by binding to the VDR, leading to heterodimerisation of the receptor with the retinoid X receptor. This complex then binds to the vitamin D response element in the promoter region of target genes, and together with transcription factors and co-regulator molecules alters gene expression (Brancaccio *et al.* 2007; Reddy *et al.* 2010). The vitamin D receptor also activates CYP3A, a cytochrome P450 enzyme that detoxifies lithocholic acid in the liver and intestine (Makishima *et al.* 2002) which could explain the relationship with colorectal cancer.

The evidence surrounding an effect of vitamin D on all of the aforementioned outcomes is uncertain. For cancer, a few meta-analyses have been conducted of prospective studies (Yin *et al.* 2013; Maalmi *et al.* 2014; Kim *et al.* 2014) which suggest a modest association between 25-OH-D concentrations and risk of cancer, but a much stronger association between serum 25-OH-D and risk of cancer mortality. For CVD, meta-analyses of observational studies suggest a significant reduction in CVD risk with higher serum 25-OH-D concentrations (Grandi *et al.* 2010; Parker *et al.* 2010; Wang *et al.* 2012b). Cross-sectional analyses have found strong associations between 25-OH-D concentrations and BMI and obesity (Brock *et al.* 2010), however, a meta-analysis of 12 RCTs found that the obesity measures body weight, body mass index (BMI), fat mass, percentage fat mass or lean body mass were not influenced by the final vitamin D status reached, or the change in 25-OH-D from baseline (Pathak *et al.* 2014). Bolland *et*

al. conducted a vitamin D supplementation trial sequential meta-analysis looking at several outcomes including myocardial infarction or ischaemic heart disease, stroke or cerebrovascular disease, and cancer for which there were 9, 8 and 7 RCTs included, respectively (Bolland *et al.* 2014). Thresholds were calculated using a 15% risk reduction which was thought to represent the smallest effect that would be clinically relevant for an individual. Vitamin D supplementation alone or with calcium did not change the relative risk (RR) of any of the aforementioned outcomes by 15% or more; for all studies with or without calcium, the RRs (95% CI) were 1.02 (0.93, 1.13) for myocardial infarction or ischaemic heart disease, 1.01 (0.90, 1.13) for stroke or cerebrovascular disease, and 0.99 (0.93, 1.05) for cancer.

1.1.9.3 Vitamin D and mortality

A few meta-analyses assessing the effect of vitamin D supplementation on mortality have been conducted (Chowdhury *et al.* 2014; Zheng *et al.* 2013; Bjelakovic *et al.* 2014). One of these, by Bjelakovic *et al.* was conducted as part of a Cochrane review. This included 56 RCTs, 48 of which were in healthy participants, and most of which were in women over 70 y of age (Bjelakovic *et al.* 2014). The mean supplementation length was 4.4 y and vitamin D supplementation was found to decrease mortality risk (RR, 95% CI 0.97, 0.94-0.99). Zheng *et al.* conducted another meta-analysis of 42 RCTs which suggested that vitamin D supplementation was protective against all-cause mortality if given for longer than 3 y (RR, 95% CI 0.95, 0.90-0.98), but was not protective if taken for less than 3 y (RR, 95% CI 1.04, 0.97-1.12) (Zheng *et al.* 2013). The RCTs included in this were mostly in older adults aged over 60 y with an age range of 37-89 y. Supplementation was predominantly in the form of vitamin D_3 and ranged from 5 μg/d to 5000 μg/wk, although most studies gave at least 20 μg/d and many gave a much higher dose. Trials in adults with chronic kidney disease and heart failure were included, as were those which gave calcium in addition to vitamin D (Zheng *et al.* 2013). The above two meta-analyses and another by Chowdhury *et al.* (Chowdhury *et al.* 2014) all found D_3 to have a greater effect on mortality compared with D_2. Chowdury *et al.* included data from 22 RCTs (14 and 8 trials on the effects of D_3 and D_2 respectively) and found that the risk of all-cause mortality was 11% lower with D_3 supplementation (RR, 95% CI 0.89, 0.80-0.99), but there were no significant effects of D_2 supplementation (RR, 95% CI 1.04, 0.97-1.11). However, it can be seen that the D_2

confidence intervals overlap those for D_3, so no conclusions can be inferred on the inferiority of D_2. The Cochrane review included 38 trials that reported effects of D_3, but only 12 that reported the effect of D_2. It found significant reductions in mortality after D_3 supplementation (RR, 95% CI 0.94, 0.91-0.98) but not D_2 (RR, 95% CI 1.02, 0.96-1.08) (Bjelakovic *et al.* 2014). Comparison of doses of D_2 <20 μg/d (RR, 95% CI 0.82, 0.17-3.98) with doses ≥20 μg/d (RR, 95% CI 1.02, 0.95-1.10) revealed no differences but confidence intervals were large and only one D_2 study used a dose of <20 μg/d. For D_3, although there were no significant differences between the effects of the high and low doses, doses of <20 μg/d showed a reduction in mortality (RR, 95% CI 0.92, 0.87-0.97) whereas doses ≥20 μg/d did not (RR, 95% CI 0.96, 0.92-1.00) (Bjelakovic *et al.* 2014). In summary, it seems that there may be a risk-reducing effect of vitamin D on mortality in the older population, but in order to determine whether this effect occurs solely in the elderly, and whether only D_3, or both forms have an effect, more well-conducted RCTs are needed, particularly those using D_2. Furthermore, the majority of RCTs have used high dose supplementation and there are fewer studies examining the effect of doses in line with recommendations.

1.2 Cardiovascular Disease

The focus of this thesis is the non-calciotropic effects of vitamin D and their relationship to CVD. A common feature to most CVD is atherosclerosis and the consequences of atherosclerotic plaque rupture leading to vessel occlusion or aneurism resulting in haemorrhage. The sequence of events in atherosclerosis are depicted in **Figure 1.5** (Frayn 2005; Libby *et al.* 2011). Atherosclerosis develops over many decades without any overt symptoms and the clinical horizon where CVD events occur is usually in the fifth decade of life and beyond. It is nodular hardening of large to medium size arteries that is characterised by the accumulation of lipids derived from low density lipoprotein cholesterol (LDL-C) in the sub-endothelial intima. Picture **a** shows a normal healthy artery which consists of three layers; the tunica intima is the inner layer facing the blood which contains smooth muscle cells (SMCs) and is lined by a monolayer of endothelial cells (Libby *et al.* 2011). The middle layer, the tunica media, is also made up of SMCs and these are embedded in an extracellular matrix. The adventitia is the outer layer which contains mast cells, nerve endings and microvessels. Initially, in atherosclerosis (picture **b**), leukocytes (mostly monocytes) in the blood

adhere to the endothelial cells and migrate into the intima layer along with LDL-C particles which become oxidised (Libby *et al.* 2011; Sherer *et al.* 2006). In the intima, monocytes differentiate into macrophages that take up oxidised LDL-C and become foam cells (Sherer and Shoenfeld 2006). Accumulation of foam cells in the arterial wall forms fatty streaks which are the first visible stage in atherosclerosis (Frayn 2005). Apoptosis of foam cells appears to be a critical phase in the development of atherosclerotic plaques. As the atherosclerotic lesion progresses (picture **c**), SMCs in the media migrate into the intima via the actions of matrix-metalloproteinase-9 (MMP-9) and other proteinases which help degrade the extracellular matrix. Proinflammatory cytokines and growth factors produced by foam cells result in proliferation of these SMCs (Packard *et al.* 2008) which secrete extracellular matrix macromolecules including collagen and elastin. As the atherosclerotic lesion advances, some macrophages and SMCs in the plaque die by apoptosis and a lipid core forms as extracellular lipid from these dead or dying cells builds up in the centre of the plaque. If the fibrous cap of the plaque remains firm it does not cause acute damage, only obstruction to blood flow (Frayn 2005). Alternatively, the cap can be weakened and made rupture prone. For example, inflammatory cytokine production can stimulate macrophages to express collagenases which break down collagen in the fibrous cap. It can also lead to apoptosis of the plaque's smooth muscle cells which are important for the repair and maintenance of the fibrous cap (Libby 2001). Furthermore, macrophages in the vessel wall secrete metalloproteinases (such as MMP-9) and cathepsins which can destabilise the fibrous cap. If the cap fractures (picture **d**) a thrombus forms as coagulation components in the blood make contact with tissue factors inside the plaque, and this has the potential to suddenly obstruct arterial blood flow {Sherer and Schoenfeld 2006; Borissoff *et al*. 2011}.

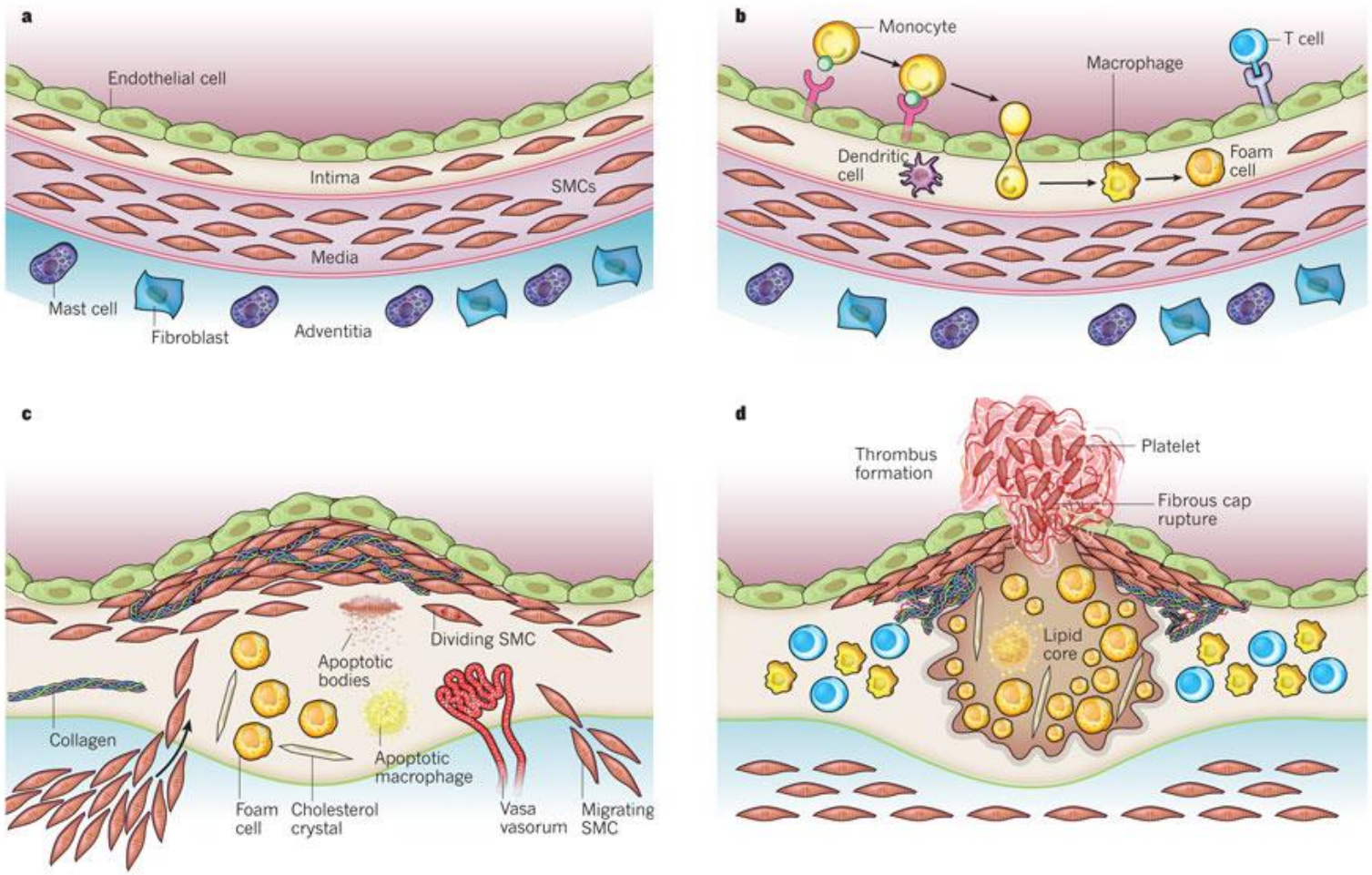


Figure 1.5 Development of an atherosclerotic lesion taken from (Libby *et al.* 2011). SMC, smooth muscle cell.

1.3 Vitamin D and Cardiovascular Disease

Ecological data was initially the basis for suggesting a potential association between vitamin D and CVD. Higher latitudes experience greater CVD mortality than lower latitudes (Zittermann *et al.* 2005), there is an inverse relationship between altitude and CVD mortality (Mortimer *et al.* 1977), and there is a greater CVD mortality in the winter months compared to late summer and early autumn (Douglas *et al.* 1991). In the UK, CVD prevalence is generally higher in the north of the UK especially the rural areas of Scotland compared with southern England (Elford *et al.* 1989). Scragg *et al.* hypothesised in 1981 that this variation in CVD mortality could be due to variations in UV exposure which influences production of vitamin D, and that vitamin D may be having a protective effect against CVD (Scragg 1981). However, it could also be argued that cold weather increases risk of CVD as it increases BP (Brennan *et al.* 1982). Since then, observational studies have found inverse associations between vitamin D status and cardiovascular outcomes/CVD risk (Kendrick *et al.* 2009; Kim *et al.* 2008; Kilkkinen *et al.* 2009). For example, the third National Health and Nutrition Examination Survey (NHANES) (1988-1994) that included 16,603 men and women aged >18 y found that participants with 25-OH-D deficiency (defined as serum concentrations <50 nmol/L) had an increased risk of prevalent CVD (OR 1.20, 95% CI 1.01-1.36) (Kendrick *et al.* 2009) comapred to those who were not deficient. In addition, Anderson *et al.* prospectively analysed data from an electronic medical records database for 41,504 subjects and found strong associations between serum 25-OH-D concentrations and incident heart failure, coronary artery disease/myocardial infarction (all $P<0.0001$) and stroke ($P = 0.003$) (Anderson *et al.* 2010). Several systematic reviews of observational studies have been published (Grandi *et al.* 2010; Parker *et al.* 2010; Pittas *et al.* 2010; Wang *et al.* 2012b). Wang *et al.* conducted the most recent of these in which 19 prospective studies up until February 2012 were included, with 6123 CVD cases in 65,994 participants (Wang, *et al.* 2012b). Comparing the lowest study-defined 25-OH-D category with the highest, the pooled relative risks (95% CIs) for total CVD, CVD mortality, CHD and stroke were 1.52 (1.30-1.77), 1.42 (1.19-1.71), 1.38 (1.21-1.58) and 1.64 (1.27-2.10) respectively. A regression analysis determined that the increment in CVD risk with decreasing 25-OH-D concentrations was generally linear for serum 25-OH-D levels between 20 and 60 nmol/L, but that above 60 nmol/L there were only a few data points and no clear increase or decrease in CVD risk could be detected (Wang

et al. 2012b). Two other meta-analyses that only included studies up until 2009 found a greater risk of incident CVD, CVD mortality (Grandi *et al.* 2010), and prevalent cardiometabolic disorders (Parker *et al.* 2010; Grandi *et al.* 2010) in participants with lower 25-OH-D concentrations. An umbrella review of the above meta-analyses summarised the findings and showed that the greatest effect of serum 25-OH-D concentrations was on CVD mortality compared to prevalent and incident CVD (Theodoratou *et al.* 2014). However, despite these observational findings, causality cannot be assumed.

Although there have been relatively few trials investigating the effect of vitamin D supplementation on CVD, three meta-analyses of RCTs have been conducted (Bolland *et al.* 2011; Bolland *et al.* 2014; Myung *et al.* 2013). Bolland *et al.* reanalysed data from the Women's Health Initiative Calcium/Vitamin D supplementation (WHI CaD) trial, a 7 year study in which 36282 community dwelling postmenopausal women were randomly assigned to placebo or a calcium and vitamin D supplement (containing 500 mg calcium and 5 μg vitamin D_3) twice daily (Bolland *et al.* 2011; Hsia *et al.* 2007). Data from this trial were combined with that from two other trials (Grant *et al.* 2005; Lappe *et al.* 2007) in a meta-analysis which showed that calcium and vitamin D increase the risk of myocardial infarction (RR = 1.21, *P*=0.04) and stroke (RR = 1.20, *P*=0.05) (Bolland *et al.* 2011). Myung *et al.* (Myung *et al.* 2013) conducted a meta-analysis of RCTs that as well as trials of only vitamin D, also included those that gave other vitamins (Mark *et al.* 1996) or calcium in addition to vitamin D (Brazier *et al.* 2005; Hsia *et al.* 2007; Berggren *et al.* 2008; Prince *et al.* 2008). After the inclusion of 7 RCTs (Mark *et al.* 1996; Komulainen *et al.* 1999; Trivedi *et al.* 2003; Brazier *et al.* 2005; Hsia *et al.* 2007; Berggren *et al.* 2008; Prince *et al.* 2008), it was concluded that there was no effect of vitamin D supplementation on major CVD events (RR 1.02, 95% CI 0.98, 1.07). This was the case for low dose (2 trials) and high dose (5 trials) supplementation, whether vitamin D was given singly (2 trials) or in combination with other supplements (5 trials), and for the outcomes CVD mortality (3 trials), MI (2 trials), and stroke (5 trials) (Myung *et al.* 2013). However, in the two meta-analyses described above, it is not possible to disaggregate the calcium/vitamin D effects. If calcium has a negative effect on CVD, it may be masking a potential protective effect of vitamin D. The most recent meta-analysis of RCTs giving vitamin D supplementation with or without calcium ran

analyses to assess the effect of vitamin D alone (this included studies that had given the same calcium supplement to both treatment groups) and vitamin D with or without calcium. For both, there were no significant results for MI or ischaemic heart disease, or for stroke or cerebrovascular disease. When all trials were combined, the relative risks (95% CI) were 1.02 (0.93, 1.13) for the former outcome (nine trials, 48,647 patients) and 1.01 (0.90, 1.13) for the latter outcome (eight trials 46,431 patients) (Bolland *et al.* 2014).

In summary, the observational evidence suggests an association between vitamin D status and CVD, but the evidence from meta-analyses of RCTs does not show a benefit of vitamin D supplementation on CVD.

1.4 Vitamin D and CVD risk factors/factors associated with the development of CVD

It is difficult and costly to study CVD as an endpoint in primary prevention because of the large number of people and the long follow-up period required. Furthermore, it is uncertain what dose level should be chosen. This is important because if the level is too low it will lack efficacy and if it is too high there is a risk of toxicity. As a prelude to any intervention it is worthwhile assessing the effects of vitamin D on surrogate markers of CVD risk rather than disease endpoints. Such an approach is more feasible and also facilitates the investigation of possible mechanisms by which vitamin D may influence CVD risk. The following section reviews the effects of vitamin D status on CVD risk factors and factors associated with the development of CVD.

1.4.1 Established risk factors and factors associated with the development of CVD

There are several well-established and conventional CVD risk factors including hypercholesterolaemia, dyslipidaemia (atherogenic lipoprotein phenotype), elevated BP, and type 2 diabetes which is preceded by insulin resistance. HsCRP, an acute phase reactant, and fibrinogen, a plasma glycoprotein involved in clot formation, have also been shown to be important in predicting risk of CVD (Torres and Ridker 2003; Kaptoge *et al.* 2012; Danesh *et al.* 2005).

1.4.1.1 Lipids

The progression of atherosclerotic disease is mediated by circulating blood lipids (Quinn *et al.* 1987; Frostegard *et al.* 1990). Initial studies investigating effects on CVD risk have focused on elevated total cholesterol (TC) and LDL-C. Familial hypercholesterolaemia which is an autosomal dominant inherited disorder affecting 1:500 people is associated with a hugely increased risk of CVD under the age of 50 y (Austin *et al.* 2004). More moderate elevations of total and LDL-C are prevalent in the general population over the age of 50 in the UK and confer substantial risk. However, low concentrations of high density lipoprotein (HDL-C) are also strongly associated with risk (Boden 2000) and the Prospective Triallist Collaboration found the ratio of total cholesterol:HDL-C (TC:HDL) ratio in particular to be up to twice as informative as other lipid metrics of CVD (Lewington *et al.* 2007).

Cross-sectional studies have suggested an association between vitamin D and HDL-C (Maki *et al.* 2009) and triglycerides (Ford *et al.* 2005; Hypponen *et al.* 2008), but two meta-analyses of randomised trials suggest little, or no effect of vitamin D supplementation on lipid fractions (Elamin *et al.* 2011; Wang, *et al.* 2012a). Elamin *et al.* included 11-12 randomised trials which predominantly gave D_3 as the form of supplementation and were mostly in older adults. Not every study was placebo-controlled, but all had a comparison group that was not receiving the intervention. No significant differences between treatment groups were found for changes in total cholesterol (TC), triglycerides, LDL-C or HDL-C (P=0.25-0.91). (Elamin *et al.* 2011). Another meta-analysis by Wang *et al.* was conducted a year later in 2012 (Wang *et al.* 2012a). This found LDL-C to significantly increase with vitamin D supplementation (pooled mean net change 0.084 mmol/L, 95% CI 0.014, 0.153) and this was strongest in studies in obese people (0.090 mmol/L, 95% CI 0.004, 0.099) and those of less than 1 y duration (0.097 mmol/L, 95% CI 0.015, 0.179). No treatment effects were found for TC, HDL-C or triglycerides, apart from in studies lasting longer than 1 y for which there was a significant reduction in HDL-C concentrations (-2.01 mg/dl, -3.83 to -0.18). A total of 12 RCTs with data on 1346 participants were included in this meta-analysis and the age range was 18-80 y, although the majority of studies were in older adults. Most studies (7) gave D_3, only one used D_2, and the remaining studies supplemented with alpha-calcidiol or calcitriol. Study duration was between 42 d and 3 y and daily doses ranged

between 7.5 and 83 μg/d, but the majority of studies gave pharmacological doses such as a weekly dose of 1000 μg D_3 (Jorde *et al.* 2010a). However, the heterogeneity of the studies included in the meta-analysis, and their lack of power to detect a change in lipid concentrations mean the evidence is inconclusive regarding an effect of vitamin D supplementation on lipids (Wang *et al.* 2012b).

1.4.1.2 Blood pressure

BP is linearly associated with risk of CVD (Lewington *et al.* 2007). Most studies report clinic BP, which is measured seated with the subject at rest to yield systolic (SBP) and diastolic (DBP) BP. British Hypertension Society Guidelines recommend taking three measures, discarding the first and taking the average of the two (O'Brien *et al.* 2001), but BP can vary during the day (Mancia *et al.* 2007). More recent guidelines recommend that if clinic BP is more than a SBP/DBP of 140/90 mm Hg, 24 h ambulatory BP (ABP) monitoring should be used to confirm the diagnosis of hypertension as a day-time average SBP/DBP ≥ 135/90 mm Hg (National Institute for Health and Clinical Excellence 2011). 24 h ABP has been shown to be better at predicting cardiovascular risk compared with clinic BP (Mancia *et al.* 2007; Staessen *et al.* 1999). Furthermore, evidence has demonstrated that it is able to more accurately measure the size of reduction in BP brought about by a treatment compared to clinic BP, due to the results being more reproducible over time (Coats *et al.* 1992), and the unlikelihood of a 'white coat' or placebo effect (Mancia *et al.* 2007; Mancia *et al.* 1995).

Many observational studies have investigated a possible association between vitamin D status and BP and their results have been summarised in a meta-analysis by Burgaz *et al.* which included 4 prospective and 14 cross-sectional studies. All studies either diagnosed hypertension using office BP or used self-reported data; they did not measure ABP (Burgaz *et al.* 2011). The pooled odds ratio (OR) for hypertension in participants in the highest 25-OH-D category compared with those in the lowest category was 0.73 (95% CI 0.63, 0.84) (Burgaz *et al.* 2011).

A number of RCTs have looked at changes in BP, primarily as seated BP in response to vitamin D supplementation, and shown mixed results (Asemi *et al.* 2013b; Forman *et*

al. 2013; Jorde *et al.* 2010a; Muldowney *et al.* 2012; Nagpal *et al.* 2009; Witham *et al.* 2010; Wood *et al.* 2012). Only a few trials have used ABP monitors (Judd *et al.* 2010; Larsen *et al.* 2012; Witham *et al.* 2013c; Witham *et al.* 2014) (**Table 1.4**) and these were in participants who had hypertension (Witham *et al.* 2013c; Witham *et al.* 2014; Judd *et al.* 2010; Larsen *et al.* 2012) or had previously suffered from a stroke (Witham *et al.* 2012). None showed a significant difference in the change in BP between treatment and placebo groups after treatment with vitamin D_3 (Judd *et al.* 2010; Larsen *et al.* 2012; Witham *et al.* 2012; Witham *et al.* 2013c; Witham *et al.* 2014) or vitamin D_2 (Witham *et al.* 2012). Several meta-analyses of RCTs investigating the effect of vitamin D supplementation on BP have been published (Pittas *et al.* 2010; Elamin *et al.* 2011; Witham *et al.* 2009; Wu *et al.* 2010; Kunutsor *et al.* 2014). Most of these have concluded that there is no significant effect of vitamin D on SBP or DBP (Pittas *et al.* 2010; Elamin *et al.* 2011; Kunutsor *et al.* 2014). However, one meta-analysis that included 8 RCTs found a significant decrease in DBP (-3.1 mm Hg, 95% CIs –5.5 to -0.6), (Witham *et al.* 2009). By contrast, Wu *et al.* in a meta-analysis of 4 RCTs observed that vitamin D supplementation decreased SBP by 2.44 mm Hg (Weighted mean difference -2.44, 95% CI -4.86, -0.02), but had no significant effect on DBP (Wu *et al.* 2010). Both these meta-analyses were limited by the small size of the studies included and significant heterogeneity between studies. Kunutsor *et al.* performed the most recent meta-analysis of 16 studies aiming to investigate the effects of oral vitamin D supplementation alone and found no significant treatment effects; weighted mean differences (95% CIs) were -0.94 (-2.98, 1.10) mm Hg and -0.52 (-1.18, 0.14) mm Hg for SBP and DBP, respectively (Kunutsor *et al.* 2014).

Although the above demonstrates that the evidence is uncertain with regards to an association between vitamin D and BP, mechanisms have been suggested for a possible link between the two. It has been shown that vitamin D in its active form ($1,25(OH)_2D$) is a negative endocrine regulator of the renin-angiotensin system (RAS) (Li *et al.* 2004) which is a regulatory cascade essential for the long-term control of BP, electrolyte levels, and volume homeostasis (Jeunemaitre *et al.* 1992; Li 2003). Renin is a rate-limiting component of this system which acts to cleave angiotensin I (Ang I) from angiotensinogen, and it is suppressed when there is an increase in $1,25(OH)_2D$ concentrations. Angiotensin-converting enzyme converts Ang I to Ang II, which causes

blood vessels to constrict, resulting in an increased BP (Li 2003; Li *et al.* 2004). Li *et al.* found a several-fold increased expression of renin and production of angiotensin II in VDR-null mice. They also found evidence that this led to hypertension and cardiac hypertrophy (Li *et al.* 2002). Another possible mechanism for an effect of vitamin D on BP is via changes in PTH. PTH has been found in vascular endothelium and smooth muscle cells (Fitzpatrick *et al.* 2008) which would allow for it to have direct regulatory effects on the vessel wall (Larsen *et al.* 2012). Further, observational studies have found a positive association between parathyroid hormone and both SBP and DBP (Snijder *et al.* 2007), and PTH intravenous infusion has been shown to increase BP in normal subjects (Hulter *et al.* 1986). A different suggested mechanism is via direct effects of $1,25(OH)_2D$ on the vasculature. This is supported by the expression of 1α-hydroxylase in endothelial cells and evidence that $1,25(OH)_2D$ may affect vascular smooth muscle cell growth (Carthy *et al.* 1989).

Table 1.4 Summary of clinical trials investigating the effect of vitamin D supplementation on 24 h ambulatory blood pressure (24 h ABP).

Study	Population Characteristics	Treatment groups	No. of participants completed	Mean age y	Duration of study	Mean baseline and follow-up 25-OH-D in nmol/L		Effect of treatment *vs.* control
						Treatment	Control	
Judd *et al.* (2010) (Pilot study)	Black or African American adults with 25(OH)D 25 - 75 nmol/L, and SBP 130 - 150 mm Hg.	5000 µg vitamin D_3 weekly for 3 wk, 0.5 µg $1,25(OH)_2D$ twice daily for 1 wk, or placebo	7	47	3 wk	46 in $1,25(OH)_2D$ group and 24 in D_3 group	27	No treatment effect for D_3 group. $1,25(OH)_2D$ group experienced a 9% decrease in mean SBP compared with placebo (P<0.001) but this was not sustained 1 wk after conclusion of $1,25(OH)_2D$ therapy
Larsen *et al.* (2012).	Hypertensive adults with 24h SBP ≤150 mm Hg and/or 24 h DBP ≤95 mm Hg	75 µg/d vitamin D_3 or placebo	112	61	20 wk	57 to 110	57 to 50	No significant treatment effects. Between group differences: SBP -3 mm Hg (*P*=0.26), DBP -1 mm Hg (*P*=0.18)
Witham *et al.* (2012)	Stroke patients	Single oral dose of 2500 µg D_2 at baseline or placebo	55	67	16 wk	59 to 51	38 to 40	No significant treatment effects at 8 wk or 16 wk. At 8 wk between group differences: -0.7 mm Hg (95% CI -5.5, 4.2) for SBP and -1.1 mm Hg (95% CI -4.1, 1.8) for DBP
Witham *et al.* (2013c)	Older patients with SBP > 140 mm Hg and DBP < 90 mm Hg	2500 µg D_3 every 3 mo or placebo	142	77	1 y	45 to 70	45 to 52	No significant treatment effects. Between group differences: SBP 0 mm Hg (95% CI -2 ,2), DBP 0 mm Hg (95% CI -1, 2)
Witham *et al.* (2014)	Patients with hypertension resistant to conventional treatment	2500 µg vitamin D_3 every 2 mo or placebo	61	63	6 mo	41 to ?	42 to ?	No significant treatment effects. Between group differences: SBP +3 mm Hg (95% CI -4 ,11), DBP -2 mm Hg (95% CI -6, 2)

1.4.1.3 Insulin resistance

Insulin resistance is associated with increased CVD risk (Rader 2007); it is characterised by elevated fasting insulin concentrations and is strongly associated with obesity (Kahn *et al.* 2000). Several large epidemiological studies have found 25-OH-D concentrations to be associated with improved insulin resistance and sensitivity (Chiu *et al.* 2004; Kayaniyil *et al.* 2010; Liu *et al.* 2009). However RCTs have found mixed results; some have found a significant beneficial effect of supplementation (Nagpal *et al.* 2009; Nikooyeh *et al.* 2011; von Hurst *et al.* 2010) whilst others have shown no effect (Jorde *et al.* 2010a; Patel *et al.* 2010). A RCT in 100 apparently healthy, but centrally obese men observed an improvement in postprandial insulin sensitivity measured by the oral glucose insulin sensitivity index (OGIS) after supplementation with 3000 μg D_3 once a fortnight for 6 wk (mean difference in change in OGIS between placebo and the D_3 group was 41.1 ± 15.5, P=0.01) (Nagpal *et al.* 2009). Similarly, Nikooyeh *et al.* conducted a 12 wk D_3 supplementation trial in 90 diabetic subjects and showed a significant decrease in the homeostasis model assessment of insulin resistance (HOMA-IR) after 25 μg/d D_3 compared to placebo (P<0.001). In contrast to these findings, a RCT of 438 overweight or obese subjects found no significant difference in insulin sensitivity in the fasting state from baseline to 1 y between the placebo group and two D_3 groups that received either 500 μg or 1000 μg/wk (Jorde *et al.* 2010a). Furthermore, 10 or 30 μg/d D_3 did not lead to significant improvements in insulin sensitivity in a 4 month RCT of patients with type 2 diabetes (Patel *et al.* 2010).

A meta-analysis has been published to summarise the effects of vitamin D supplementation on glycaemic control and insulin resistance (George *et al.* 2012). For insulin resistance, 11 studies were included, 4 of which were in individuals with normal fasting glucose and 6 in individuals with abnormal glucose tolerance. When all 11 studies were combined there was no effect of vitamin D supplementation on insulin resistance; the standard mean difference favouring vitamin D supplementation was -0.07 (95% CIs -0.20, 0.06). This was also the case when looking at only patients with normal fasting glucose, but in patients with diabetes or impaired glucose tolerance, there was a small improvement in insulin resistance (standard mean difference favouring vitamin D supplementation -0.25, 95% CI -0.48, -0.03) (George *et al.* 2012).

Several mechanisms have been suggested as to how vitamin D may affect insulin sensitivity and release. Receptors for $1,25(OH)_2D$ and 1α-hydroxylase have been found in pancreatic β cells (Bland *et al.* 2004) and $1,25(OH)_2D$ may either directly alter insulin action at the level of the adipocyte, or increase insulin release from pancreatic β cells (Teegarden *et al.* 2009). Vitamin D may also influence insulin resistance through its effects on PTH. PTH has been implicated in impaired insulin release from β cells (Fadda *et al.* 1990) and it may cause a decrease in insulin signalling and reduced insulin sensitivity (Teegarden and Donkin 2009). Alternatively, insulin secretion may be effected by changes in the balance between extracellular and intracellular pools of calcium as insulin secretion is a calcium dependent process and vitamin D influences the absorption of calcium from the small intestine (Pittas *et al.* 2007b).

1.4.1.4 C-reactive protein

Inflammation plays an important pathogenic role in all stages of atherosclerosis (Ross 1999). Currently, the best available inflammatory biomarker is C-reactive protein (CRP), an acute phase reactant which is a reliable, independent predictor of the risk of myocardial infarction (MI), stroke and cardiovascular death (Torres and Ridker 2003). CRP is a member of the pentraxin family as it is made up of a central pore surrounded by 5 symmetrically arranged non-covalently associated protomers. It is produced in the liver and is primarily under the control of interleukin-6 (IL-6) which increases its hepatic synthesis and secretion (Jialal *et al.* 2004). In endothelial cells, CRP has been shown to decrease nitric oxide production (Venugopal *et al.* 2002). Other effects in monocyte-macrophages include the induction of tissue factor secretion (Cermak *et al.* 1993), increased production of reactive oxygen species (Tebo *et al.* 1991) and increased release of proinflammatory cytokines (Ballou *et al.* 1992).

Cross-sectional studies have shown conflicting results as to whether vitamin D has an effect on CRP levels with some showing an inverse association between the two (Amer *et al.* 2012; Ngo *et al.* 2010), some finding that adjustment for adiposity (Hypponen *et al.* 2010) or BMI (Jorde *et al.* 2007) removed initial inverse associations, and others finding no association (Michos *et al.* 2009). Clinical trials also show inconsistent results; some find no effect of supplementation on CRP levels (Asemi *et al.* 2013a; Belenchia *et al.* 2013; Jorde *et al.* 2010b; Muldowney *et al.* 2012; Pittas *et al.* 2007a; von Hurst *et al.*

2010; Wood *et al.* 2012; Yiu *et al.* 2013; Zittermann *et al.* 2009), but decreases in CRP have been found following vitamin D supplementation in others (Asemi *et al.* 2013b; Timms *et al.* 2002; Witham *et al.* 2013b). Witham gave patients with a history of MI 2500 µg D_3 or placebo at baseline, 2 months and 4 months and found that at 6 months CRP had decreased significantly in the D_3 group compared to placebo (-1.3 *vs.* 2.0 mg/L, *P*=0.03) (Witham *et al.* 2013b). Similarly, a significant decrease in high sensitivity C reactive protein (hsCRP) concentrations was observed after 9 wk of 10 µg/d D_3 (-1.41 ± 0.55 mg/L), compared to placebo (1.50 ± 0.94 mg/L) (*P* = 0.01) in 48 pregnant women aged 18 to 40 y at week 25 of gestation (Asemi *et al.* 2013b). In contrast, two larger placebo-controlled RCTs that supplemented type 2 diabetic patients with 125 µg/d vitamin D_3 for 12 wk (Yiu *et al.* 2013), or overweight and obese subjects with 1000 or 500 µg D_3/wk for 1 y (Jorde *et al.* 2010b), found no significant difference in the change in hsCRP between groups. After smaller vitamin D_3 doses of 10 or 25 µg/d for 1 y (Wood *et al.* 2012), or 5, 10 or 15 µg/d for 22 wk in healthy young and older adults (Muldowney *et al.* 2012), hsCRP levels were unaffected. Whilst there is significant heterogeneity between published trials due to differences in baseline hsCRP concentrations, dose of vitamin D and duration of the trials, a meta-analysis has found that vitamin D supplementation significantly reduces hsCRP by 1.08 mg/L (95% CI -2.13, -0.03) (Chen *et al*. 2014). This included 10 RCTs in 924 participants published between 2009 and 2014. The median dose of vitamin D was 100 µg/d with a range of 10 to 179 µg/d. Participants who had higher baseline hsCRP levels of ≥5 mg/L had a greater reduction of 2.21 mg/L (95% CI -3.50, -0.92) in hsCRP after supplementation.

Mechanisms as to how vitamin D may affect CRP levels are uncertain, but experimental studies have shown that treatment with $1,25(OH)_2D$ inhibits the production of several other pro-inflammatory cytokines such as interleukin – 6 (IL-6) (Talmor *et al.* 2008; Muller *et al.* 1992) and tumour necrosis factor-α (TNF-α) (Muller *et al.* 1992; Cohen-Lahav *et al.* 2007) and down-regulates IL-10 expression via the binding of VDR to the promoter region of the IL-10 transcription start site (Matilainen *et al.* 2010). In mice models, deficiency of vitamin D and the VDR has been shown to lead to the acceleration of certain autoimmune diseases such as inflammatory bowel disease (IBD) (Cantorna *et al.* 2004) suggesting that vitamin D may modulate the expression of cytokine genes which are controlled by the VDR.

1.4.1.5 Fibrinogen

Fibrinogen is involved in a coagulation cascade that helps to arrest bleeding in response to vascular injury, but when disrupted can lead to thrombosis and bleeding (Borissoff *et al.* 2011). It is an acute phase protein and it is thought that high concentrations may directly promote atherogenesis or thrombosis, or increase the risk of thrombosis though effects on platelet aggregation, viscosity, fibrin (an insoluble protein essential in the clotting process and formed from fibrinogen by the action of thrombin), and/or clot deformability (Meade 1997). In 1964, experiments using pig's plasma demonstrated that fibrinogen was an essential cofactor in platelet aggregation (Born *et al.* 1964), and since then, human studies have found positive associations between plasma fibrinogen concentrations and platelet aggregation (Lowe *et al.* 1979; Meade *et al.* 1985) which may promote platelet thrombus formation (Lowe 1995). Fibrinogen is a large, elongated molecule that increases blood viscosity, and studies looking at arterial disease as an outcome that have shown associations with plasma fibrinogen have also shown an association with plasma and blood viscosity (Lowe *et al.* 1993; Yarnell *et al.* 1991). Large prospective studies such as the Northwick Park Heart Study (NPHS) and Prospective Cardiovascular Munster (PROCAM) Study have found fibrinogen concentrations to be strongly and independently associated with a higher risk of CVD death and fatal and non-fatal ischaemic heart disease (Meade *et al.* 1980; Meade *et al.* 1986; Heinrich *et al.* 1994), and this finding has been supported further by a meta-analysis of 31 prospective studies which found that for every 1g/L increase in usual fibrinogen, the age- and gender- adjusted hazard ratio was 2.42 (95% CI 2.24, 2.60) for CHD, 2.06 (95% CI 1.83, 2.33) for stroke, and 2.76 (95% CI 2.28, 3.35) for other vascular mortality (Danesh *et al.* 2005).

Several cross-sectional studies have investigated whether vitamin D is associated with fibrinogen concentrations in humans and found inconsistent results. Some have found significant inverse associations between the two (Parikh *et al.* 2012; Targher *et al.* 2006; Landin-Wilhelmsen *et al.* 1995) whilst others (Shea *et al.* 2008; Hypponen *et al.* 2010), including the Framingham Offspring Study (Shea *et al.* 2008) of 1381 subjects found no association (Hypponen *et al.* 2010). There are few published trials investigating the association between vitamin D and fibrinogen. Bjorkman *et al.* in 2009 conducted a RCT in which 218 long-term hospital inpatients aged over 65 y were

randomised to receive 10 µg/d or 30 µg/d vitamin D_3, or placebo for 6 months. A significant increase in 25-OH-D concentrations was found in the supplementation groups, but the changes in fibrinogen were insignificant (Bjorkman *et al.* 2009). Similarly a 12 wk trial in 90 type 2 diabetes patients found no significant differences in the change in fibrinogen between groups that received 25 µg/d D_3 or placebo in a yoghurt drink (*P*=0.357) (Neyestani *et al.* 2012). There is a clearly a need for further studies to investigate the potential association between vitamin D and fibrinogen.

1.4.2 Novel factors associated with the development of CVD

Other factors have more recently been found to be strongly predictive of cardiovascular events including endothelial function measured using flow mediated dilation (FMD) of the brachial artery, and arterial stiffness, most reliably measured by determining the carotid-femoral aortic pulse wave velocity ($PWV_{c\text{-}f}$) (Green *et al.* 2011; Yeboah *et al.* 2007; Mattace-Raso *et al.* 2006; Terai *et al.* 2008). Furthermore, there is evidence that MMP-9, which is involved in the pathogenesis of atherosclerotic lesions (Siefert *et al.* 2012) may be a novel marker of CVD mortality in patients with coronary artery disease (Blankenberg *et al.* 2003) and that Factor VII (FVII), which together with tissue factor initiates the coagulation cascade (Borissoff *et al.* 2011) is associated with an increased risk of CVD mortality (Meade *et al.* 1986). The possible association that vitamin D may have with the above factors will be discussed in this section.

1.4.2.1 Markers of vascular function

The relatively small number of clinical trials that have been conducted to investigate the effect of vitamin D supplementation on endothelial function and arterial stiffness in people without pre-existing CVD are summarised in **Table 1.5**. Sections 1.4.2.1.1 and 1.4.2.1.2 will provide more detail on these markers and their potential associations with vitamin D.

Table 1.5 Summary of clinical trials investigating the effect of vitamin D supplementation on endothelial function measured using flow mediated dilation (FMD) and arterial stiffness measured as pulse wave velocity (PWV) in individuals without pre-existing CVD.

Study	Population Characteristics	Treatment groups	No. of participants completed	Mean age y	Duration of study	Baseline and follow-up 25-OH-D (nmol/L)†		Relevant outcomes	Effect of treatment *vs.* control
						Treatment	Control		
Dong *et al.* (2010)	Normotensive adolescents (boys and girls)	Experimental group receiving 50 µg D_3/d or control group receiving 10 µg/d	44	16	16 wk	33 to 86	34 to 60	Arterial stiffness	↓ PWV (7.6%)
Gepner *et al.* (2012)	Post-menopausal women with serum 25-OH-D 25-150 nmol/L	Placebo or 62.5 µg D_3/d	109	64	4 mo	30 to 46	32 to 32	FMD, PWV	None
Harris *et al.* (2011)	Overweight African-American men and women	Placebo or 1500 µg monthly oral vitamin D_3	45	30	16 wk	34 to 101	38 to 49	FMD	↑ FMD (3.1%)
Larsen *et al.* (2012)	Hypertensive men and women	Placebo or 75 µg vitamin D_3/d	112	61	20 wk	23 to 44	23 to 20	PWV	None
Sugden *et al.* (2008)	Men and women with type II diabetes mellitus and serum 25-OH-D <50 nmol/l.	Placebo or single oral dose of 2500 µg vitamin D_2.	34	64	8 wk	40 to 63	36 to 44	FMD	↑ FMD (2.3%)

Study	Population Characteristics	Treatment groups	No. of participants completed	Mean age y	Duration of study	Baseline and follow-up 25-OH-D (nmol/L)†		Relevant outcomes	Effect of vitamin D *vs.* control
						Treatment	Control		
Tarcin *et al.* (2009)	Healthy men and women	Treatment group with 25-OH-D<25 nmol/l 7500 µg D_3 intra-muscularly monthly for 3 mo, or control group with 25-OH-D ≥ 75 nmol/L no supplementation	46	23	3 mo	20 to 117	75 to ?	FMD	↑ FMD (3.4%)‡
Witham *et al.* (2010)	Men and women with type 2 diabetes and baseline 25-OH-D<100 nmol/l	Placebo, or two treatment groups receiving a single dose at baseline of either 2500 µg or 5000 µg D_3	58	66	16 wk	41 to 59 in the 2500 µg group and 48 to 76 in the 5000 µg group	45 to 53	FMD	None
Witham *et al.* (2013a)	Healthy South Asian women with baseline 25-OH-D<75 nmol/L	Placebo or single oral dose of 2500 µg D_3 at baseline	49	41	8 wk	27 to ?. Sig. ↑ at 8 wk in comparison to placebo (10 nmol/L, 95% CI 6, 14)	27 to ?	FMD, PWV	None
Yiu *et al.* (2013)	Men and women with type 2 diabetes and serum 25-OH-D levels <75nmol/L	Placebo or 125 µg D_3/d	99	65	12 wk	21 to 59	22 to 24	FMD, PWV	None

†Values are means. ‡Compared to baseline value.

1.4.2.1.1 Endothelial function

Disruption of the functional integrity of the vascular endothelium is an important process in the development of atherosclerosis (Bonetti *et al.* 2003). Normal functioning of the endothelium is dependent on the bioavailability of the vasodilator nitric oxide (NO) and the capacity of the vascular endothelium to synthesise NO, which appears to have both anti-thrombotic and anti-atherosclerotic properties (Bonetti *et al.* 2003). NO is produced from the amino acid L-arginine via the action of endothelial nitric oxide synthase (eNOS) expressed by the endothelium (**Figure 1.6**) (Griendling and Fitzgerald 2003). Cofactors involved in this process include calmodulin and reduced nicotinamide dinucleotide phosphate (NADPH). The release of NO activates guanylate cyclase in smooth muscle cells resulting in an increased production of cyclic guanosine monophosphate (cGMP) and a decrease in intracellular calcium (Behrendt and Ganz 2002). This has the effect of relaxing the smooth muscle cells and causing vasodilation. In endothelial dysfunction, impairment of endothelium-dependent vasodilation occurs due to a decrease in the bioavailability of vasodilators including nitric oxide (NO), and an increase in endothelium-derived contracting factors (Griendling and FitzGerald 2003). Endothelial dysfunction results in an increased production of inflammatory cytokines which lead to the initiation and progression of atherosclerotic lesions and later contribute to plaque instability and rupture (Behrendt and Ganz 2002). Endothelial function can be measured using flow mediated dilation (FMD) of the brachial artery, a method which in humans has been shown to represent endothelium-derived nitric oxide (NO) bioavailability (Green *et al.* 2014). FMD involves measuring the dilation of the brachial artery in response to occlusion-induced increased blood flow (hyperaemia) caused by deflation of a cuff on the forearm after occlusion at suprasystolic BP. It is regarded as a reliable *in vivo*, non-invasive measurement of endothelial function (Uehata *et al.* 1997; Donald *et al.* 2008) and has been found to be strongly predictive of cardiovascular events in patients at moderate to high risk of CVD (Green *et al.* 2011; Yeboah *et al.* 2007).

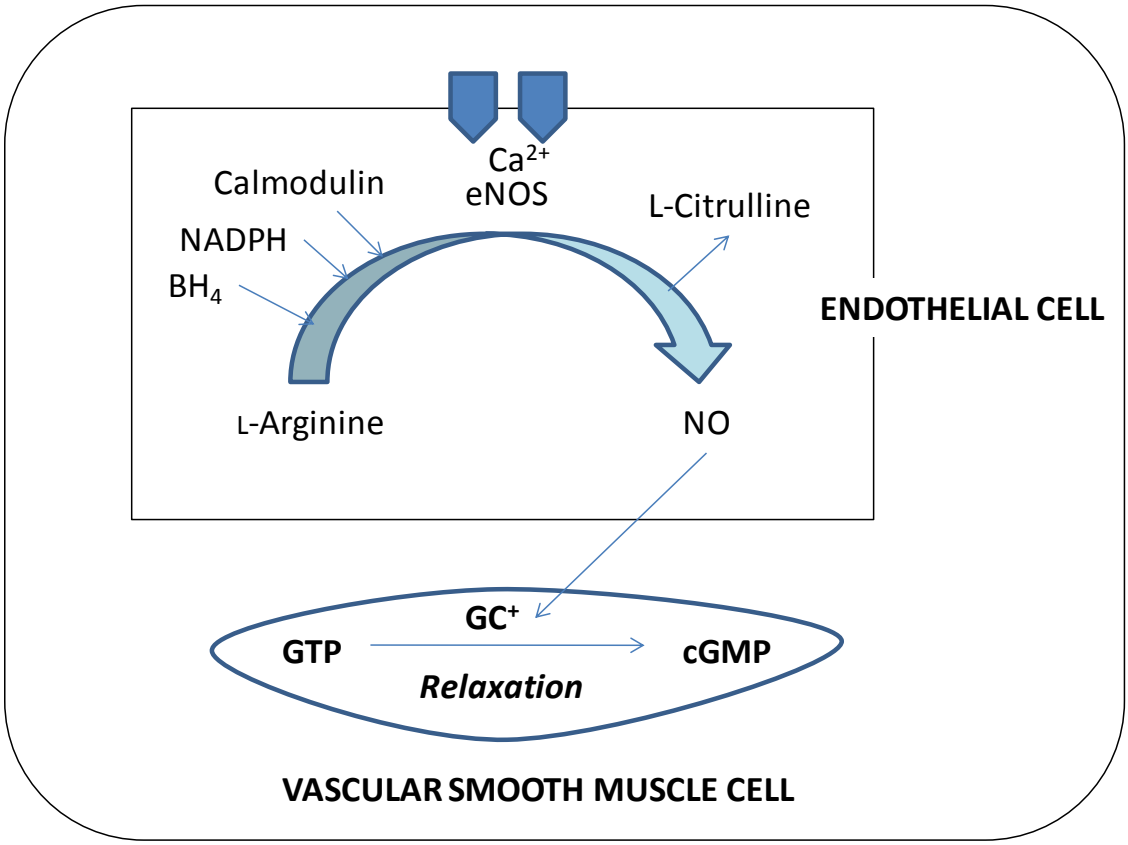


Figure 1.6 The Nitric Oxide signalling pathway adapted from (Behrendt and Ganz 2002). BH_4, tetrahydrobiopterin; cGMP, cyclic guanosine monophosphate; eNOS, endothelial nitric oxide synthase; GC, guanylate cyclise; GTP, guanosine triphosphate; NADPH reduced nicotinamide-adenine dinucleotide phosphate and NO, nitric oxide.

The association between vitamin D status and endothelial function is a relatively new area of research. In the last few years cross-sectional studies have been published which have shown lower serum levels of 25-OH-D to be associated with increased endothelial dysfunction in healthy adults (Al Mheid *et al.* 2011; Jablonski *et al.* 2011). In addition, several clinical trials have been conducted to investigate effects of vitamin D supplementation on FMD (**Table 1.5**). Some show an improvement (Tarcin *et al.* 2009; Harris *et al.* 2011; Sugden *et al.* 2008) whilst others shown no effect (Witham *et al.* 2010; Witham *et al.* 2013a; Yiu *et al.* 2013; Gepner *et al.* 2012) (**Table 1.5**). Three of these trials recruited only participants with type 2 diabetes, a group of people that have been shown to have impaired endothelial function (Guerci *et al.* 2001). Two showed no effect on FMD after 12 wk of 125 µg D_3/d (Yiu *et al.* 2013) or 12 wk after a single baseline dose of either 2500 µg or 5000 µg D_2 (Witham *et al.* 2010). In contrast, Sugden *et al.* conducted a smaller study which 34 participants completed and found

that participants in the vitamin D group experienced a significant improvement in FMD compared with placebo (2.35 *vs.* 0.06%, *P*=0.048) (Sugden *et al.* 2008). This RCT gave a single dose of 2500 μg D_3 at baseline in the winter to older participants with a mean age of 64 y and measured FMD at 8 wk. Further evidence for a positive effect of vitamin D comes from two studies in younger participants (Harris *et al.* 2011; Tarcin *et al.* 2009). In a study that was not placebo controlled, Tarcin *et al.* recruited 46 healthy men and women with a mean age of 23 y. Half of these were vitamin D-deficient (25-OH-D<25 nmol/l), and half had a mean 25-OH-D of 75 nmol/l (control group). The vitamin D deficient subjects received 7500 μg D_3 per month as intramuscular injections, and FMD measurements were significantly improved at 3 months compared with the control group (*P* = 0.001) (Tarcin *et al.* 2009). Harris *et al.* recruited 45 healthy African Americans with a mean age of 30 y and randomly allocated them to either placebo (*n*=23) or a monthly dose of 1500 μg oral vitamin D_3 (*n*=22) for 16 wk. A significant treatment x time interaction was found (*P*=0.047) and at 16 wk, FMD had significantly improved in the supplementation group (1.8 ± 1.3%) compared with the placebo group (-1.3 ± 0.6%) (Harris *et al.* 2011). Other studies in post-menopausal women (Gepner *et al.* 2012) and healthy South Asian women (Witham *et al.* 2013a) failed to show an improvement in FMD after D_3 supplementation. All of the trials mentioned above have either given participants very high doses of vitamin D daily or pharmacological doses as a single or monthly dose. To the author's knowledge, there are no trials of doses in line with dietary recommendations. The study by Tarcin *et al.* is poorly controlled and none of the studies have a duration of longer than 16 wk. Most have only measured FMD at baseline and at the end of the study, rather than taking repeated measures throughout the intervention period. Two of the studies showing an improvement in FMD were in diabetes patients (Sugden *et al.* 2008; Witham *et al.* 2010), and the findings may not be applicable to healthy participants. Whilst the cross-sectional evidence is suggestive of an association between vitamin D and endothelial function, no firm conclusions can be made, and more good quality RCTs are warranted to investigate this further.

Although much is still to be elucidated, mechanisms as to how vitamin D may affect endothelial function have been suggested through the results of cellular and animal work (Andrukhova *et al.* 2014; Molinari *et al.* 2011). Both the enzyme 1α-hydroxylase

and the VDR have been found in vascular endothelial cells (Merke *et al.* 1989; Zehnder *et al.* 2002; Jablonski *et al.* 2011) suggesting that vitamin D may modulate endothelial function via a possible autocrine mechanism whereby $1,25(OH)_2D$, produced in endothelial cells binds to the VDR to mediate endothelial effects (Merke *et al.* 1989). It has been shown in human umbilical vein endothelial cells (HUVEC) that $1,25(OH)_2D_3$ can cause a significant concentration-dependent increase in endothelial NO production via eNOS activation (Molinari *et al.* 2011). To support this, a study by Andrukhova *et al.* carried out experiments in mice carrying a mutant, functionally inactive VDR and showed that they had a lower bioavailability of NO as a result of a reduced expression of eNOS (Andrukhova *et al.* 2014).

1.4.2.1.2 Arterial stiffness

Arterial stiffness is determined by structural and functional components of the artery (Arnett *et al.* 1994). Collagen and elastin are two important scaffolding proteins which are present in the vascular wall; collagen provides strength and allows the artery to cope with high pressure, and elastin allows for reversible extensibility of the artery (Wagenseil and Mecham 2012). Their relative contribution determines the stability, resilience and compliance of the vascular wall. Normally these proteins are kept in a stable state, but several factors can lead to an overproduction of abnormal collagen and decreased quantities of normal elastin (**Figure 1.7**). Elastin can be degraded by proteases such as matrix-metalloproteinases (MMPs) and damaged in aging and tissue injury (Wagenseil and Mecham 2012). This can result in the production of more collagen fibres which are 100-1000 times stiffer than elastic fibres, and therefore the mechanical properties of the artery are shifted into the stiffer range. Calcium deposits, which increase significantly with age, can build up in the media of large arteries and also increase the stiffness of the arterial wall (Wagenseil and Mecham 2012). Furthermore, additional cross-linking on the elastin and collagen fibres by advanced glycation end-products (AGEs) can increase the stiffness of the fibres. AGEs increase with age and form crosslinks in more places along the collagen and elastin fibres compared with normal cross-linking (Wagenseil and Mecham 2012). In addition to a lower ratio of elastin to collagen, an increase in smooth muscle tone or smooth muscle cell hypertrophy contributes to an increase in vascular stiffness (Zieman *et al.* 2005; Arnett *et al.* 1994).

Arterial stiffness can be estimated using pulse wave velocity (PWV) which involves dividing the path distance between two arterial recording sites by the transit time of the arterial pulse wave; the faster the PWV, the stiffer the artery. Carotid-femoral PWV (PWVc-f), based on estimating the velocity of the pressure wave travelling between the carotid and femoral arterial sites, is considered to be the 'gold-standard' measurement (Shahin *et al.* 2013; Laurent *et al.* 2006). Arterial stiffness is a strong predictor of cardiovascular events, particularly in older participants (Mattace-Raso *et al.* 2006; Terai *et al.* 2008). A systematic review of 16 cohort studies included 17,635 participants, 1,785 of which had a CVD event in the follow-up period. The pooled age- and gender- adjusted hazard ratio per 1-SD change in $\log_e$ aortic PWV was 1.45 (95% CI 1.30, 1.61) for CVD (Ben-Shlomo *et al.* 2014).

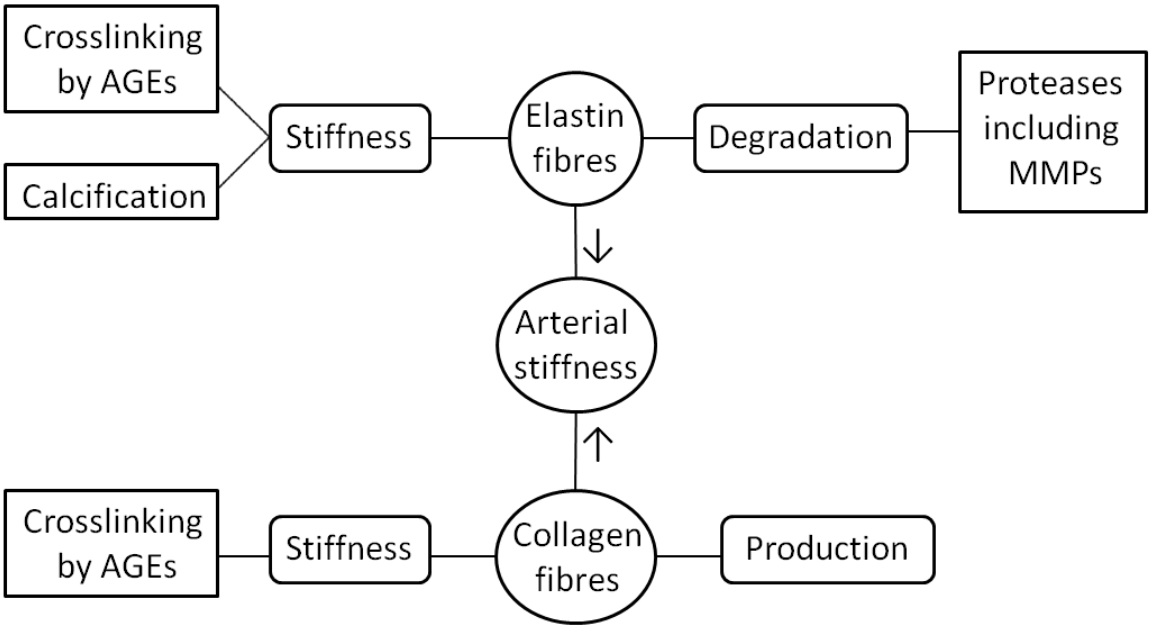


Figure 1.7 Factors that increase vessel wall stiffness adapted from (Wagenseil and Mecham 2012). AGE, advanced glycation end-products; MMPs, matrix-metalloproteinases

Many cross-sectional studies have shown inverse associations between vitamin D and arterial stiffness (Al Mheid *et al.* 2011; Andrade *et al.* 2008; Giallauria *et al.* 2012; Lee *et al.* 2012; Lieberman *et al.* 2013; Pirro *et al.* 2012; Seker *et al.* 2013; Webb *et al.* 2012; Zagura *et al.* 2011; Rezai *et al.*). However, there are relatively few clinical trials that have investigated the effect of vitamin D supplementation on arterial stiffness

(**Table 1.5**). Only three have been in healthy subjects; one in adolescents (Dong *et al.* 2010), one in postmenopausal women (Gepner *et al.* 2012), and the other in South Asian women (Witham *et al.* 2013a). The study in adolescents randomised subjects aged 14-18 y to either a control group (10 µg vitamin D_3/d) or an experimental group (50 µg vitamin D_3/d) for 16 wk. A significant time x treatment interaction was observed for PWVc-f measured using applanation tonometry (SphygmoCor). PWV significantly increased in the control group from 5.38 ± 0.53 m/sec to 5.71 ± 0.75 m/sec, but decreased in the experimental group from 5.41 ± 0.73 m/sec to 5.33 ± 0.79 m/sec. However, this study was flawed in that it did not have a placebo group and was only investigator-blinded (Dong *et al.* 2010). Gepner *et al.* conducted another study in which 114 postmenopausal women were randomised to 62.5 µg vitamin D_3 daily or placebo for 4 months. This was a well-conducted study, but it showed no significant differences between groups in the change in PWV (P=0.65) measured using applanation tonometry (SphygmoCor). This lack of an effect on PWV may be due to the women's baseline concentrations of total 25-OH-D being quite high though (80.6 ± 26.2 nmol/L in the placebo group and 75.6 ± 26.7 nmol/L in the treatment group) (Gepner *et al.* 2012). However, an 8 wk RCT in South Asian women with mean baseline serum 25-OH-D concentrations of 27 nmol/L also found no change in PWV measured using applanation tonometry (SphygmoCor) in a group given a single oral dose of 250 µg D_3/d compared with placebo (P=0.046) (Witham *et al.* 2013a). Other trials have been in subjects with type 2 diabetes mellitus (Breslavsky *et al.* 2013; Yiu *et al.* 2013) or hypertension (Larsen *et al.* 2012). These 3 studies have all been placebo-controlled and double-blinded, but their findings cannot be extrapolated to healthy populations. Two showed no change in arterial stiffness in response to treatment (Larsen *et al.* 2012; Yiu *et al.* 2013), but the other showed arterial stiffness (Breslavsky *et al.* 2013) measured as aortic augmentation index (AI) using SphygmoCor to significantly decrease in a group receiving 25 µg/d D_3 compared with placebo (-7.0% ± 6.0 *vs.* 0.3 ± 8.7, P=0.01). The trial showing a positive effect of vitamin D supplementation was the one out of the 3 which used the lowest dose of vitamin D in the treatment group (25 µg/d compared to 75 µg/d (Larsen *et al.* 2012) and 125 µg/d (Yiu *et al.* 2013)). However, it was the study with the longest treatment period (12 months compared to 20 wk (Larsen *et al.* 2012) and 12 wk (Yiu *et al.* 2013)) and also the one with the lowest baseline levels of serum 25-OH-D (mean of 30 nmol/L compared to 57 (Larsen *et al.*

2012) and 54 (Yiu *et al.* 2013) nmol/L). It may be that trials of a longer duration and with lower baseline 25-OH-D levels are needed in order to see an improvement in arterial stiffness with vitamin D supplementation.

Mechanisms have been suggested for a potential effect of vitamin D on arterial stiffness. One possibility is via changes in calcium homeostasis. Vitamin D deficiency may lead to an increased vascular calcification which contributes to stiffening of the arteries (Watson *et al.* 1997). Other suggestions are via effects on the vascular wall by inhibition of the renin-angiotensin system as described in section 1.4.1.2 (Li *et al.* 2002) or suppression of vascular smooth muscle cell proliferation (Chen *et al.* 2010).

1.4.2.2 Matrix-metalliproteinase-9

Matrix metalloproteinases (MMPs) are a family of Zn^{2+} and Ca^{2+}-dependent proteolytic enzymes which, on activation, are responsible for the breakdown of extracellular matrix in both normal physiological processes and pathological states (Dollery *et al.* 1995). Currently in humans there are 23 known MMPs, and MMP-9 is responsible for cleaving type 4 collagen and gelatine (Baker *et al.* 2014). MMPs are involved in the pathogenesis of atherosclerotic lesions (Siefert and Sarkar 2012) and MMP-9 and MMP-3 in particular mediate a remodelling process in which the fibrous cap in atherosclerotic plaques is resorbed (matrix degradation) or redeposited (matrix synthesis). This process is in balance under stable conditions, but under proinflammatory conditions, degradation of the cap is favoured over synthesis, leading to plaque rupture (Liu *et al.* 2006). In patients with coronary artery disease, serum MMP-9 levels may represent a marker of inflammation (Ferroni *et al.* 2003). Studies have also shown that compared to normal human arteries, MMPs have an increased expression in atherosclerotic vessels, particularly within the regions of the plaque cap that have a thinner wall and are at a higher risk of rupture (Galis *et al.* 1995a). Furthermore, MMP-2 and MMP-9 in particular have been found in monocyte-derived foam cells. Secretion of these MMPs provides a continuous source of extracellular matrix degradation within the lesions, contributing to the disruption of plaques as described above (Galis *et al.* 1995b).

There are a few cross-sectional studies which have investigated whether vitamin D is associated with levels of MMP-9 (Baker *et al.* 2014; Timms *et al.* 2002; Wasse *et al.* 2011). Timms *et al.* recruited 171 healthy British adults of Bangladeshi origin aged 35-65 y and found that serum 25-OH-D concentrations independently determined plasma MMP-9 levels (r= -0.41, P<0.0001) (Timms *et al.* 2002). Similarly, in a population of 92 submariners, naval officers and support crew, MMP-9 levels were significantly and inversely correlated with 25-OH-D levels (r= -0.41, P=0.01) (Baker *et al.* 2014).

The sparse clinical trial data for the association between vitamin D and MMP-9 has shown mixed results; 2 studies have observed no treatment effect (Muldowney *et al.* 2012; Timms *et al.* 2002) and one has found a significant decrease in MMP-9 concentrations with supplementation (Shab-Bidar *et al.* 2011). Muldowney *et al.* conducted a double-blind RCT in which men and women aged 20-40 y (n=202) and ≥64y (n=192) received cholecalciferol at doses of 5, 10, or 15 µg/d of vitamin D_3, or placebo during the winter in Ireland. After 22 wk of supplementation, there was no difference between groups in the mean change in MMP-9 (P=0.91 in the younger age group and P=0.97 in the older age group) (Muldowney *et al.* 2012). The study mentioned above by Timms *et al.* also conducted an intervention study in which subjects with 25-OH-D levels <27 nmol/L (n=54) were randomly allocated into two groups which were given three monthly injections of vitamin D_3; one a high dose of 1250 µg, and the other 12.5 µg. After 1 y of supplementation, reductions in MMP-9 were similar in both groups; in the high dose group, the change was -57.84% compared to -64.68% in the low dose group (Timms *et al.* 2002). A smaller study randomly allocated 100 type 2 diabetes patients to receive a plain yoghurt drink containing 170 mg calcium and no vitamin D/250 mL or a vitamin D_3-fortified yoghurt drink containing 170 mg calcium and 12.5 µg D_3/250 mL twice a day for 12 wk. This RCT found a significant difference in the mean change in MMP-9 between groups (-2.3 ± 3.7 in the fortified yoghurt group *vs.* +0.44 ± 7.1 in the plain yoghurt group, P=0.02) (Shab-Bidar *et al.* 2011). The above demonstrates uncertainty surrounding the effect of vitamin D on MMP-9 and there is a need for further well-conducted RCTs that are powered to detect a change in MMP-9. It is not known what the mechanisms for a potential effect may be, although cellular work has found that 1,25$(OH)_2$D down-regulates and suppresses MMP-9 production (Anand *et al.* 2009; Coussens *et al.* 2009) including in

the presence of TNF-α which is a powerful inducer of MMP-9 (Bahar-Shany *et al.* 2010).

1.4.2.3 Factor VII (FVII)

FVII, like fibrinogen is involved in the coagulation cascade (Borissoff *et al.* 2011). It can be measured as FVII coagulant activity ($FVII_c$) or activated FVII ($FVII_a$). FVIIa, together with tissue factor, initiates the coagulation cascade through the extrinsic pathway (section 2.4.1) and high levels of FVII have been shown to be associated with CVD (Meade *et al.* 1986). Data from 1511 white men aged 40 to 64 y, recruited into the Northwick Park Study, found that an increase of about one SD in $FVII_c$ increased the risk of IHD death within 5 y of recruitment by 62% (Meade *et al.* 1986). Furthermore, higher baseline levels of $FVII_c$ were found in those who died of CVD compared to those who survived in the Northwick Park Study (Meade *et al.* 1980), and in those who had a coronary event after 8 y of follow-up compared to those who did not in the Prospective Cardiovascular Münster study (PROCAM) study of 2780 men (Junker *et al.* 1997). To the author's knowledge, only one study has investigated the effect of vitamin D on FVII. This cross-sectional study recruited 206 subjects and measured both $FVII_a$ and $FVII_c$, but found no significant associations between these and either serum 25-OH-D or $1,25(OH)_2D$ concentrations (Jorde *et al.* 2007). Mechanisms as to how vitamin D may affect FVII concentrations have not been suggested.

1.5 Vitamin D and cognitive function/mood

In addition to CVD, this thesis also investigates whether vitamin D supplementation can improve cognitive function in healthy older men and women. Vitamin D may have a direct effect on the brain as supported by the finding of the enzyme 1α-hydroxylase and the VDR for $1,25(OH)_2D$ in the human brain in neurons and glial cells (Eyles *et al.* 2005). This is supported by experiments in rat models which have shown that $1,25(OH)_2D$ can modulate calcium binding proteins with neuroprotective roles (Alexianu *et al.* 1998), and also the production of nerve growth factor (Neveu *et al.* 1994) and nitric oxide synthase (Garcion *et al.* 1998) in the brain.

A few meta-analyses of observational studies have shown associations between a lower vitamin D status and cognitive impairment (Etgen *et al.* 2012; Balion *et al.* 2012;

van der Schaft *et al.* 2013), executive dysfunction (Annweiler *et al.* 2013) and Alzheimer disease (Balion *et al.* 2012). One of the meta-analyses included 9 observational studies and 3 vitamin D supplementation trials (Annweiler *et al.* 2013). Cross-sectional comparisons were made between participants with a low, study-defined vitamin D status and those with a high vitamin D status. Lower 25-OH-D concentrations were associated with lower scores for episodic memory assessed using tests based on learning and retrieval of items (effect size -0.16, 95% CIs -0.25, -0.07), and worse scores on tests to assess executive function. Whilst the observational evidence summarised in these meta-analyses suggests a beneficial effect of vitamin D on cognitive performance, many of the studies included have not adjusted for important confounders such as gender, age, BMI, seasonality and education (van der Schaft *et al.* 2013) and therefore causality cannot be assumed. There are a few vitamin D supplementation trials that have been conducted with cognitive function as an outcome but these have shown mixed results and were either not randomised or placebo-controlled (Annweiler *et al.* 2012; Przybelski *et al.* 2008) or had short follow-up periods of 6 wk or less which is unlikely to be long enough to see an effect (Dean *et al.* 2011; Przybelski *et al.* 2008). Their effects on executive function were summarised in the meta-analysis by Annweiler *et al.* which compared vitamin D supplementation groups (*n*=234) to control groups (*n*=257) and found no significant difference in the change in test score between groups (effect size 0.14, 95% CIs -0.04, 0.32) (Annweiler *et al.* 2013). In addition to these studies, analysis of data from 4143 women aged ≥65 y in the Women's Health Initiative (WHI) Calcium and Vitamin D Trial and the WHI Memory Study found that supplementation over a mean follow-up of 7.8 y did not result in any treatment effects compared to placebo for domain-specific cognitive function. However, calcium as 1000 mg of calcium carbonate/d was given in addition to 10 μg D_3/d and it was not possible to separate the effects of the two (Rossom *et al.* 2012).

Similar to cognitive function, trials assessing the effect of vitamin D supplementation on mood have shown inconsistent results (Lansdowne *et al.* 1998; Harris *et al.* 1993; Jorde *et al.* 2008; Vieth *et al.* 2004b). Jorde *et al.* conducted a RCT in which 441 participants aged 21-70 y were randomised to placebo or 500 μg or 1000 μg D_3/wk for 1 y and all were also given 500 mg calcium/d. Depressed mood was assessed using a

multiple choice questionnaire. Although statistical analyses don't appear to have been conducted for a comparison between groups, which would have been the correct way of testing for a true effect of supplementation on mood, there were significant improvements in scores in the two supplementation groups, but not in the placebo group (Jorde *et al.* 2008). Another trial of 1 y duration in 250 women with a mean age of 62 y gave 277 mg calcium and 10 µg vitamin D/d to half the women and only 277 mg/d calcium to the other half. Mood was measured using a scale that measures six mood factors including Depression-Dejection, Tension-Anxiety, Anger-Hostility, Vigour-Activity, Fatigue-Inertia and Confusion-Bewilderment, but no differences between groups were found (Harris *et al.* 1993). From the above, it might be considered that high dose supplementation is needed to see an effect. However, whilst they were not placebo controlled, two trials of lower dose supplementation have found positive effects of supplementation on measures of mood and wellbeing (Vieth *et al.* 2004b; Lansdowne and Provost 1998). One of these trials by Vieth *et al.* assessed wellbeing using a short questionnaire based on conventional depression-screening tools which asked questions about energy and mood (Vieth *et al.* 2004b). A significant improvement was found in winter wellbeing scores in 51 outpatients after both 15 and 100 µg/d D_3 for a few months. Landsdowne *et al.* gave 44 healthy subjects with a mean age of 22 y 10 or 20 µg D_3/d, or no vitamin D for 5 days in the winter months. Positive Affect, measured using a self-reported measure of both positive and negative affectivity was significantly enhanced in both treatment groups compared to the control group ($P<0.001$) (Lansdowne and Provost 1998), although there were no significant effects on negative affectivity.

In summary, evidence of a relationship between vitamin D and cognitive function and mood is inconclusive and much of the evidence is of poor quality. There is a need for well-conducted, placebo-controlled and randomised trials investigating the effect of vitamin D supplementation on these outcomes.

1.6 Conclusions

The above review of the literature identifies that there are limitations and inconsistencies in the current knowledge base regarding the effect of vitamin D on CVD risk factors and markers of disease progression. This is a fairly new area of research

and relatively few vitamin D supplementation trials have been conducted. Many have either have not been placebo controlled or double-blinded, or have been under-powered (too small a sample size) to detect a change in particular outcomes. Others have not adequately controlled for potential confounding factors. Furthermore, it would seem that the majority of published trials have used high, often pharmacological doses of vitamin D which are irrelevant to real life and intakes in line with recommendations. Due to the uncertainty in the evidence, this thesis sets out to address some of these issues.

1.7 Aims and objectives of thesis

i) To investigate cross-sectional associations between serum 25-OH-D concentrations and vitamin D intake, age, BMI, physical activity, gender, season and skin pigmentation in baseline data from two previously conducted RCTs (MARINA and CRESSIDA) in healthy men and women aged 40-70 y.

ii) To assess, using data from the CRESSIDA trial, the impact on vitamin D status of advice to consume 1-2 portions of oily fish per wk.

iii) To investigate using data from the MARINA and CRESSIDA studies whether low vitamin D status measured as serum 25-OH-D concentrations is associated with a higher 24 h BP, increased arterial stiffness measured as PWV, impaired endothelial function measured as FMD and higher concentrations of the inflammatory marker hsCRP, adjusting for potential confounders.

iv) To conduct a RCT to evaluate the effect on serum 25-OH-D metabolites of intakes of 5 and 10 μg/d of vitamins D_2 and D_3, provided in a malted milk drink, in the UK winter months. The aim of this study was to help decide on the dose and form to use in a RCT investigating whether low dose vitamin D in the dietary range may affect a range of CVD risk factors and factors associated with the development of CVD. The objective was to determine whether a dose of 10 μg/d results in significantly greater increases in serum 25-OH-D concentrations compared with 5 μg/d and whether low dose vitamin D_2 supplementation results in meaningful increases in 25-OH-D concentrations as some authors have argued that D_2 is less effective than

D_3 at increasing vitamin D status, but using D_2 in the RCT investigating effects on CVD risk factors would allow differentiation between the effects of sunlight and diet as 25-OH-D_2 is produced from D_2 whereas 25-OH-D_3 is produced in response to skin UVB exposure as well as from D_3.

v) To conduct a randomised placebo controlled trial to investigate whether low dose vitamin D_2 in the dietary range provided in a malted milk drink in healthy men and post-menopausal women aged 50-70 y during the winter months will lower 24 h BP and improve endothelial function in healthy older men and women. This will also investigate whether D_2 has effects on arterial stiffness, haemostatic and inflammatory risk factors, fasting glucose, indices of insulin secretion and sensitivity and, as an exploratory outcome, cognitive function.

Chapter 2

Methods

This chapter describes the methods used to collect anthropometric, physiological and blood analyte measurements in the studies covered in the later chapters. It also provides an outline of previously run studies on which vascular function measures were made and vitamin D status later measured.

2.1 Anthropometric measurements

2.1.1 Height

Height was measured using a wall mounted stadiometer. The investigator ensured that the volunteer removed his/her shoes and was standing upright with heels and shoulders against the measuring rod, knees and back straight, and looking forward. The measuring slide was then pushed slowly onto the head, compressing the hair, so that it touched without bending. The reading was taken to the nearest 0.1 cm.

2.1.2 Weight

Participants were weighed at their initial screening visit on a Tanita body composition analyser (Tanita model BC-418). They removed any outer clothing and shoes and stepped onto the weighing platform with bare feet. Participants were asked to hold the handgrips firmly in each hand with their arms slightly out to the side, but held straight to facilitate assessment of body composition. The scales were tared to 1 kg to allow for clothing weight. Measurements at other occasions were made on calibrated scales accurate to 0.1 kg, Model 764 Seca, 40 Barn Street, Birmingham, B5 5QB).

2.1.3 Waist circumference

Waist circumference was either measured against the skin, or over light clothing so that the tape could be brought as close to the body as possible. The participant was asked to stand upright and still whilst the measurement was being taken. The tape was placed at the mid-point between the bottom of the rib cage and top of the iliac crest parallel to the floor, and the measurement read to the nearest 0.1 cm.

2.2 Physiological measurements

2.2.1 Endothelial function measured by flow mediated dilation

Endothelial function was measured using the flow mediated dilation (FMD) technique. FMD is regarded as a reliable *in vivo*, non-invasive measurement of endothelial

function (Uehata *et al.* 1997; Donald *et al.* 2008) and in humans, it represents endothelium-derived nitric oxide (NO) bioavailability (Green *et al.* 2014). It involves measuring the dilation of the brachial artery in response to occlusion-induced increased blood flow (hyperaemia) caused by deflation of a cuff on the forearm after occlusion at suprasystolic BP. The dilation of the brachial artery is measured by high resolution ultrasound scanning of the brachial artery. The hyperaemia increases shear stress parallel to the long axis of the vessel (Niebauer *et al.* 1996) which is then transduced to endothelial cells through mechanoreceptors. Consequently, the G-protein expression of phosphokinase A is increased leading to an increase in endothelial NO synthase activity which acts as a catalyst for the conversion of L-arginine to NO (Sessa 2004). The diffusion of this NO into the media leads to the activation of guanylate cyclase, which converts Guanosine-5'-triphosphate (GTP) into Guanosine monophosphate (GMP). GMP has the effect of inducing smooth muscle relaxation and therefore vasodilation. FMD is calculated as the percentage increase in brachial artery diameter from baseline to maximal dilation (Harris *et al.* 2010). Endothelial independent dilation can be estimated by the administration of glycerol trinitrate sublingually, although this was not determined.

A high resolution ultrasound system with a 7-10 MHz linear array transducer (Acuson Aspen, Acuson Corporation, Mountain View, CA, USA) (**Figure 2.1**) was used. In order to become proficient in the method, the author underwent training by an experienced ultrasonographer. Measurements were made according to the recommendations of the International Brachial Artery Reactivity Task Force Guidelines for the ultrasound assessment of endothelial-dependent FMD vasodilation of the brachial artery (Corretti *et al.* 2002). Participants were asked in advance to avoid strenuous physical activity, foods high in fat, caffeine and alcohol the day before the visit as these have been shown to influence FMD (Papamichael *et al.* 2005; Clarkson *et al.* 1999; Harris *et al.* 2010; Vogel *et al.* 1997; Agewall *et al.* 2000). To begin with, the measurement procedure was explained to the participant to help ensure that they were comfortable with it and would remain as still as possible during the measurement. In a temperature controlled room (22-26˚C) (Harris *et al.* 2010), the participant was asked to rest supine on a height adjustable bed for at least 15 min before starting the measurement. It was ensured that they were warm and comfortable by providing a blanket if necessary and

changing the position of the bed/pillow. Labelled electrocardiogram (ECG) monitors were placed under the left and right collar bone and above the hip bone on the stomach on the right hand side of the participant. A pneumatic tourniquet cuff was wrapped around the forearm with the far edge in line with the elbow crease (distal to ultrasound probe) (Betik *et al.* 2004). Ultrasound gel was applied to the upper arm just above the elbow crease and a longitudinal section of the brachial artery, 2 to 15 cm above the elbow, was scanned using the ultrasound probe. Once a clear picture of the brachial artery was obtained, the probe holder and clamp were tightened to help secure the position of the probe (**Figure 2.2**). A tape measure was used to measure the distance in cm from the edge of the cuff to the edge of the probe and this was recorded. The positions of the participant's head and hand (up/on the side, and comments) were recorded and it was ensured that the same positions were used in future measurements. Using the ultrasound machine controls, the clearest area of the brachial artery was made to fill the scan area visible on the screen. The screen cursor was placed in the centre of the brachial artery and the angle altered to be in line with the direction of blood flow. In order to confirm that an artery was being scanned and not a vein, and that the ECG was still working, it was checked that there were regular waveforms appearing at the bottom of the screen. The brightness of the screen in different sections was altered to provide the clearest possible picture of the vessel wall. In addition to the ultrasound screen, the scan picture was visible on a separate computer which had computer assistant edge detection software uploaded onto it (BrachialAnalyser, Medical Imaging Applications, LCC, Iowa, USA). The participant's anonymous ID was entered into the program and the software set up to record a total of 220 frames at a speed of 0.333/s, i.e 20 frames per min for 11 min in total. The images were recorded continuously throughout, including 1 min at baseline. After baseline, reactive hyperaemia was induced by the quick inflation of the pneumatic tourniquet cuff to a pressure of 250 mm Hg for 5 min, followed by quick release and the recording of frames for another 5 min. Throughout the recording period, small movements of the probe could be made using a micrometer attached to the clamp in order to improve the clarity of the vessel walls. After the test, the image was saved before unclamping the probe and informing the participant that they were able to move again. Post-visit, the clearest section of the brachial artery (region of interest, or ROI) was chosen and selected and the edge detection software calculated the

diameter at that section for each frame (**Figure 2.3**). It is important that the diameter measurements occur at the same time in the cardiac cycle for each frame. End diastole is used, rather than end systole, as the size of systolic expansion is affected by the vessel compliance and possibly factors such as aging and hypertension. The software detects end diastole using the onset of the R-wave from the integrated ECG output (Corretti *et al.* 2002) (**Figure 2.4**). It was determined at which point after cuff release the maximum dilation occurred, by looking at frames recorded between 30 seconds and 2 min after frame 120, when the cuff was released (Corretti *et al.* 2002) (**Figure 2.5**). The 20 frames during the baseline measurement and the frames at maximum dilation were studied to check that the software was picking up the correct part of the arterial wall. Occasionally, the walls in the ROI were blurred in parts where the probe had moved slightly. Where it seemed obvious to the eye where the wall actually was, but the software had not picked up the correct part, manual adjustments were made to correct for this. Most of the time, the luminal side of the intima was selected, but occasionally this was not clear enough in the frame and the software would select the intima side of the media wall. In this case, it was ensured that the same wall was used for repeated measures later on in the study. Frames that were so unclear it was felt an accurate estimation of the arterial wall diameter could not be made, were rejected. Averages were worked out for all of the good quality frames from the first 20 frames and for the 3 largest diameter measurements at maximum dilation. FMD was calculated as the percentage increase in brachial artery diameter from baseline to maximal dilation (Corretti *et al.* 2002). For later measurements in the study on the same participant, the probe was placed the same distance away from the cuff as for the baseline measurement. Additionally, the baseline scan was brought up on the computer to act as a comparison in order to find the same section. Training data from the author are displayed in **Table 2.1**. The within person coefficient of variation (CV) for the baseline artery diameter was 3.6% and for FMD% was 19.5%.

Figure 2.1 Ultrasound machine, scan table and bed for participant to lie on.

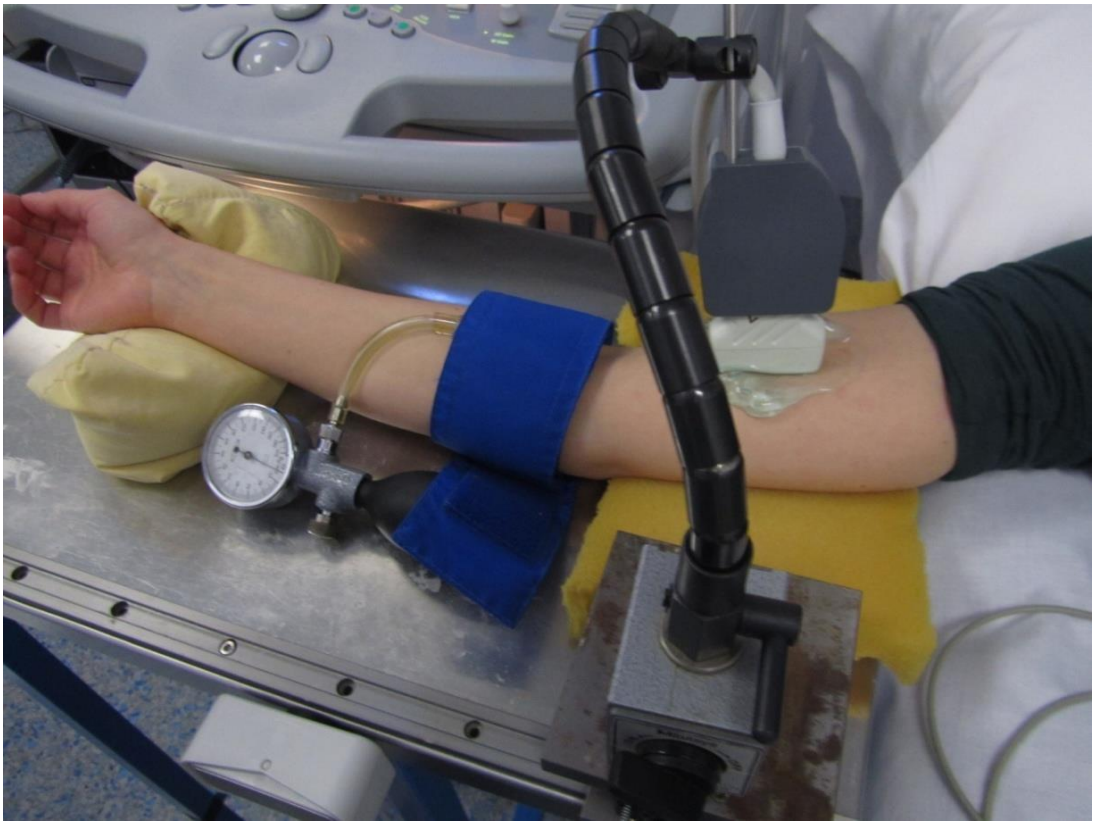


Figure 2.2 Ultrasound probe in probe holder and clamped in place ready for FMD.

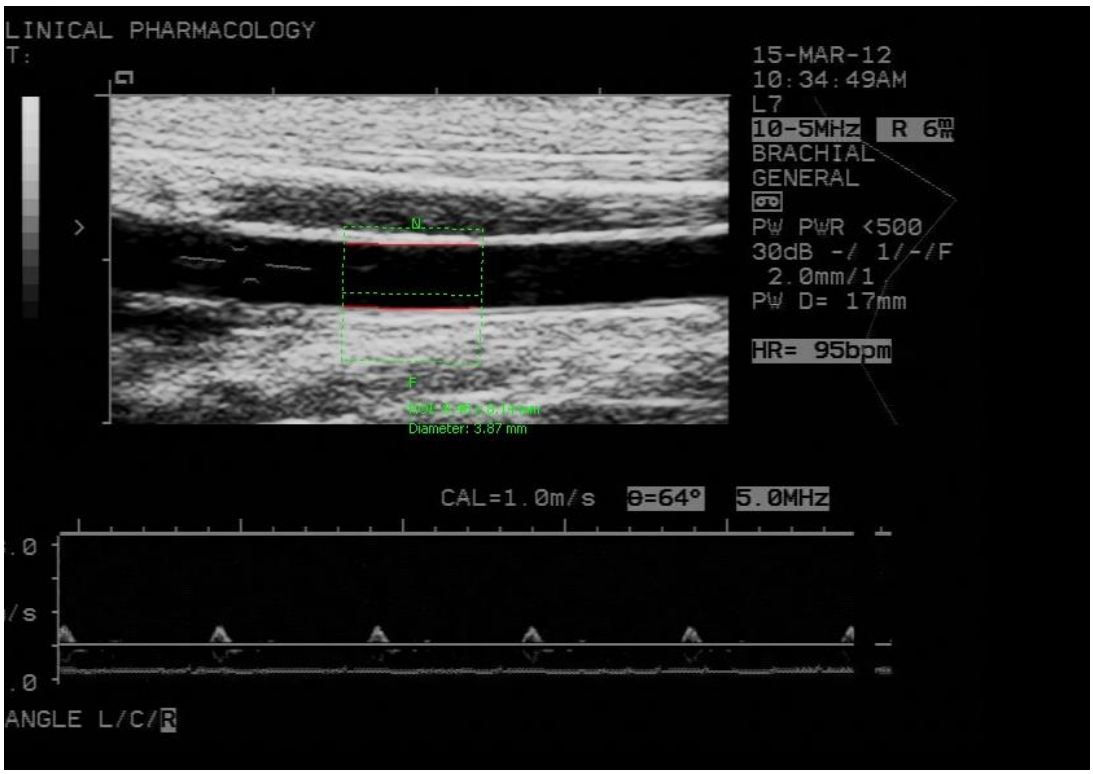


Figure 2.3 Ultrasound scan showing brachial artery (green lines represent researcher-selected region of interest (ROI), and red lines represent software detected anterior and posterior walls).

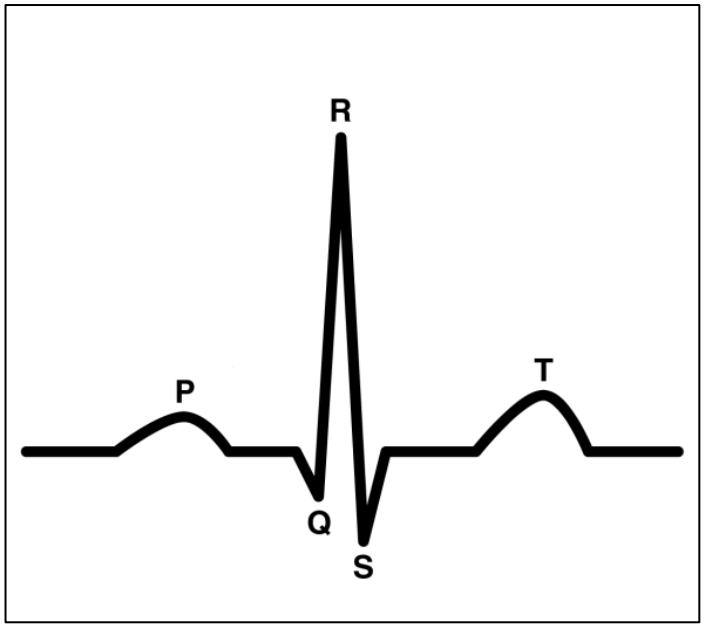


Figure 2.4 Normal ECG cardiac cycle output. Artery diameter measured at onset of R-wave.

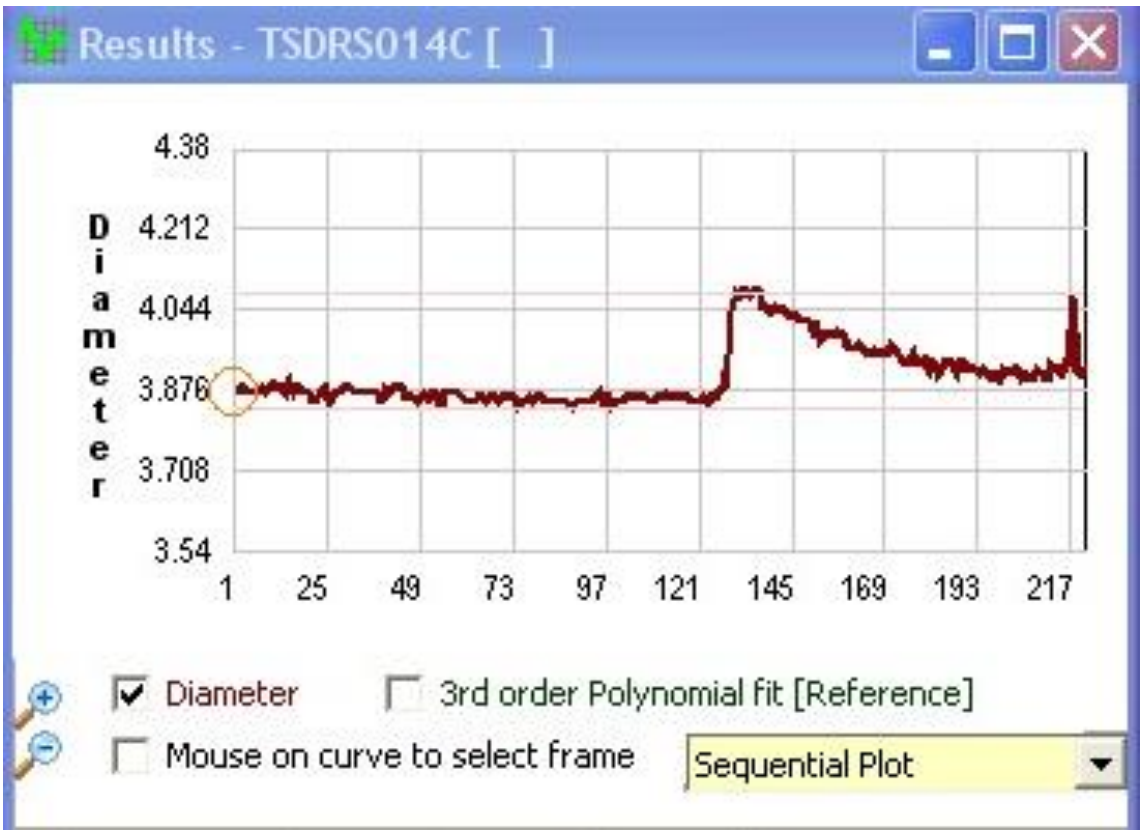


Figure 2.5 Output from Brachial Analyser showing change in brachial artery diameter over the 11 min recording period. The cuff was tightened at frame 20 and released at frame 120.

Table 2.1 Author FMD training data on 7 volunteer participants (2 measurements taken on separate days)

n	First measurement			Second measurement			Mean BAD	SD BAD	CV % BAD	Mean FMD%	SD FMD %	CV % FMD %
	BAD	Max AD	% FMD	BAD	Max AD	% FMD						
1	2.74	3.03	10.58	2.69	3	11.52	2.715	0.035	0.013	11.050	0.665	0.060
2	4.3	4.51	4.88	4.19	4.3	2.63	4.245	0.078	0.018	3.755	1.591	0.424
3	2.67	2.82	5.62	2.87	3.1	8.01	2.77	0.141	0.051	6.815	1.690	0.248
4	2.3	2.47	7.39	2.53	2.69	6.32	2.415	0.163	0.067	6.855	0.757	0.110
5	4.67	4.69	0.43	4.54	4.56	0.44	4.605	0.092	0.020	0.435	0.007	0.016
6	3.68	3.83	4.08	3.87	4.1	5.94	3.775	0.134	0.036	5.010	1.315	0.263
7	3.24	3.45	6.48	3.04	3.32	9.21	3.14	0.141	0.045	7.845	1.930	0.246
								Average CV%	3.6%		Average CV%	19.5%

BAD, baseline artery diameter; AD, artery diameter; SD, standard deviation; CV, coefficient of variation.

2.2.2 Arterial stiffness

Large artery stiffness can be estimated using pulse wave velocity (PWV) which involves dividing the path distance between two arterial recording sites by the transit time of the arterial pulse wave; the faster the PWV, the stiffer the artery. Carotid-femoral pulse wave velocity ($PWV_{c\text{-}f}$) is widely regarded as the 'gold-standard' measurement for arterial stiffness (Shahin *et al.* 2013). It is computed from the time delay between the upstroke of the arterial pressure wave at the carotid and femoral arteries and the anatomical carotid to femoral distance. It can be measured using several different devices including SphygmoCor (ArtCor Medical, Sydney, Australia), the Complior System® (Colson, Les Lilas, France) and, most recently, the Vicorder (Skidmore Medical, Bristol, UK) (Laurent *et al.* 2006; Shahin *et al.* 2013; Jatoi *et al.* 2009; Rajzer *et al.* 2008). The SphygmoCor method is regarded as the 'gold-standard' method and involves measuring carotid to femoral transit time use a tonometer which is applied to the carotid and femoral arteries. This technique requires some skill to be reproducible and the major difficulty is locating the femoral pulse on the inside of the thigh which makes it intrusive (Hickson *et al.* 2009). The Vicorder method has been developed to be used by less skilled operators and is less intrusive for the participant. It uses two cuffs placed on the leg and around the neck to determine the transit time. Hickson et al. obtained similar results using the Vicorder compared with the SphygmoCor based on analysis of 122 subjects with an average age of 53 y (Hickson *et al*. 2009). Data were available from the MARINA (Modulation of Atherosclerosis Risk by Increasing dose of N-3 fatty Acids) study (Sanders *et al.* 2011) (section 2.6.2) in which measurements had been made in a larger number of participants (335 male and female non-smoking adults aged 45-70 y) using both SphygmoCor and Vicorder techniques. **Figure 2.6** shows a comparison of the results of the two methods. The Vicorder resulted in higher values than the SphygmoCor but the slope was close to 1.0. If participants were divided into quintiles according to the method used, the Vicorder technique classified 80% of the participants into the correct or adjacent quintile (**Table 2.2**). The error associated with the measurement using the Vicorder technique was significantly lower (P<0.001) at 3.0% (SD 2.4) than that for the SphygmoCor technique (8.7%, SD 4.1) for triplicate measurements. It was decided, therefore, to use the Vicorder device.

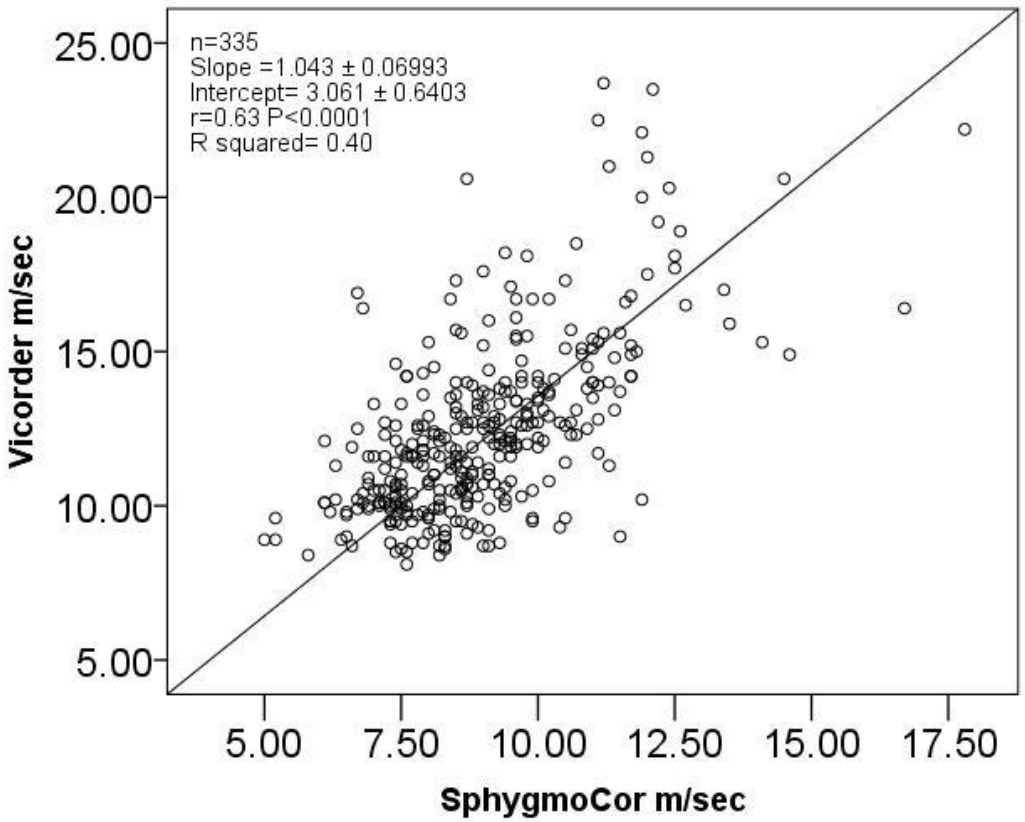


Figure 2.6 Correlation between SphygmoCor and Vicorder measurements of carotid to femoral pulse wave velocity in the MARINA study in 45-70 y old men and women (*n* = 335).

Table 2.2. Percentage of participants in each quintile of PWV measured by SphygmoCor and Vicorder in the MARINA study in 45-70 y olds (*n* = 335).

		Vicorder Quintile				
		1	**2**	**3**	**4**	**5**
SphygmoCor Quintile	**1**	38%	38%	16%	4%	4%
	2	39%	15%	33%	8%	6%
	3	15%	31%	17%	25%	12%
	4	6%	12%	28%	39%	16%
	5	5%	3%	9%	23%	60%

2.2.2.1 Vicorder

Measurements were made by the author or other trained personnel who were blinded to the treatment allocation. Initially, the measurement procedure was explained to the participant to help ensure that they were comfortable with it and would remain as still as possible during it. The participant was asked to rest supine on a height adjustable bed for at least 10 min before starting the measurement. After 10 min rest, BP was recorded once on the upper left arm (unless requested to be the right arm by the participant), using an OMRON 705CPII or equivalent automatic upper arm BP monitor, positioned with the cuff sensor over the brachial artery. Red and blue Vicorder cables were attached to the neck and thigh cuffs, respectively. The carotid pulse was found and the neck cuff placed carefully and gently around the participant's neck with the inflatable section directly over the pulse. The thigh cuff was placed tightly around the participant's right thigh, ensuring that it was as close to the femoral artery as possible (**Figure 2.7**). In order to estimate the difference in path length between the carotid and femoral arteries, the distance between the surface markings of the suprasternal notch and the top of the thigh cuff was used. Other distances were also measured as there is some debate as to which measurement to use (Van Bortel 2006). These included carotid pulse to suprasternal notch, suprasternal notch to femoral pulse (estimated location), and neck cuff to top of thigh cuff. All distances were measured in the supine position keeping the tape measure as straight as possible. The bed was then raised to a 45˚ angle. The participant's ID, gender, date of birth, BP, suprasternal notch to thigh cuff measurement, and the proximal (carotid)/distal (femoral) pulse points used were entered into the Vicorder software. The participant was informed that the measurement was about to begin, and the recording was started. Once the trained researcher had decided that a clear picture had been obtained of both arterial waveforms, the recording was stopped (**Figure 2.8**). If a clear picture could not be obtained, the recording was stopped after a short period to avoid any discomfort to the participant. Adjusting the pillow to lift the neck slightly, or raising the bed a little more usually enabled clear waveforms to be found. Carotid to femoral transit time (TT) and PWV values were calculated by the software. Training data from the author are displayed in **Table 2.3**. The within person coefficient of variation (CV) was 2.4%.

Figure 2.7 Vicorder cuffs attached to volunteer and output shown on laptop

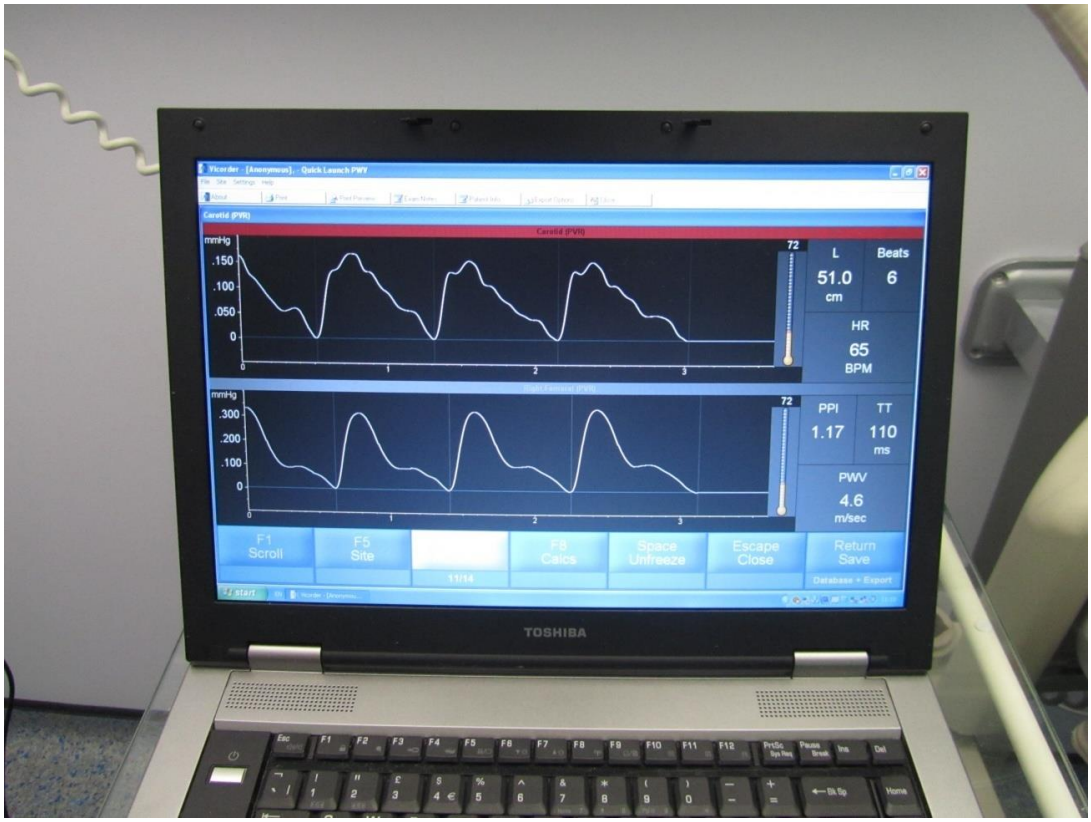


Figure 2.8 Example of a good waveform output from both Vicorder cuffs simultaneously

Table 2.3 Author Vicorder training data on 10 volunteer participants (means and SDs of 3 PWV measurements taken consecutively)

Participant	Mean (m/s)	SD	CV (%)
1	5.0	0.06	1.2
2	4.5	0.12	2.6
3	5.7	0.00	0.0
4	5.4	0.15	2.8
5	5.0	0.06	1.1
6	5.1	0.23	4.6
7	5.3	0.10	1.9
8	4.7	0.12	2.5
9	4.9	0.21	4.3
10	4.5	0.15	3.4
		Average CV (%)	2.4

2.2.2.2 SphygmoCor

In the CRESSIDA (**C**ardiovascular risk **RE**duction **S**tudy: **S**upported by an **I**ntegrated **D**ietary **A**pproach) (section 2.6.1) and MARINA (section 2.6.2) studies, PWV was measured using the non-invasive SpygmoCor apparatus (ArtCor Medical, Sydney, Australia) and SphygmoCor analysis software (SphygmoCor version 7.01 AtCor Medical Pty, Australia). Measurements were made by the study groups. Subjects were rested supine for 15 min and fitted with 3 ECG monitoring electrodes on their chest before BP was recorded supine using an automated sphygmomanometer (Omron 70CP). The distance from the femoral artery to suprasternal notch was measured by finding the pulse in the femoral. After ensuring that the ECG trace was of good quality with a prominent R wave, a probe (applanation tonometer) was used to obtain a pulse waveform from the carotid and then the femoral artery. The software calculated transit time and PWV. In order for the results to be acceptable, it was ensured that the coefficient of variation was less than 5% for 3 measurements, with no more than 6 measurements being made.

2.2.3 Blood pressure

BP and heart rate measurements were taken using an A & D UA-779 auto upper arm BP monitor that met the A/A standard of the British Hypertension Society. Participants were allowed to rest quietly at a comfortable room temperature for 10 min before the measurements were performed to help ensure that they were calm and relaxed.

During the measurement, the participant was asked to not talk and to sit quietly with their legs uncrossed and feet flat on the floor. The arm from which the measurement was being taken was comfortably supported at heart level. The appropriate sized cuff (normal or large) was selected and the bladder of the cuff placed 2-3 cm above the elbow crease. The cuff was placed snugly around the arm, with the tube positioned off-centre toward the inner side of the arm in line with the little finger. It was ensured that there was only enough space for a finger between the participant's arm and the cuff. The start button was pressed, and on completion of the measurement, the readings for DBP, SBP and heart rate were recorded. At screening, measurements were made in triplicate at 2-5 min intervals. For these triplicate measures, the first reading was discarded and the mean taken of the two following readings according to the British Hypertension Society Guidelines (O'Brien *et al.* 2001). BP measurements at visits from baseline onwards were made alone.

2.2.4 Ambulatory blood pressure (ABP)

Measurements of 24 h ambulatory BP were made using A&D TM-2430 (A & D Instruments) devices in accordance with UK guidelines (O'Brien *et al.* 2005). These have been given A/A grading and have been approved for clinical use by the British Hypertension Society (Williams *et al.* 2004). At the end of the screening, baseline, mid-point and end-point visits, participants were fitted with the ABP monitor by a trained researcher. Using the ABP monitor after the screening visit allowed the subject to familiarise themselves with it and learn how to use it before the study started. Their upper arm circumference was measured and they were provided with a suitable left arm cuff (standard size if circumference was 20-31 cm, large size if circumference was 28-36 cm), a brief instruction card (**Appendix 4**) and a diary card (**Appendix 5**). Participants who were left-handed were given a right-handed cuff. The cuff was fitted so that the yellow mark was over the brachial artery and the lower edge of the cuff was about 1 inch above the inside of the elbow. It was fitted snugly, but with a large enough gap to fit two fingers underneath. The air hose was positioned over the shoulder and the carrying case positioned on the right hand side of the participant (if right-handed) using a belt or strap, depending on the participant's preference (**Figure 2.9**). The participant was instructed to stop what they were doing and relax their arm whilst a measurement took place. They were asked to complete the diary card

following each measurement to report what they were doing immediately prior to the measurement (lying down, sitting, standing or walking plus any other details or problems that may have occurred) and to record when they went to bed and woke up. The ABP was programmed to take measurements every 30 min during the day (0700-2200) and hourly at night (2200-0700). The trained researcher nearly always fitted the monitor, and recorded the monitor number in the case report form. In a small number of cases, it was inconvenient for the participant to wear the monitor immediately after the visit (this was not the case for any screening visits). If this occurred, it was checked that the participant had a friend or relative who could fit the monitor for them when convenient with the help of the instruction card. The trained researcher also went over with the participant the important points to remember in fitting the monitor correctly, and demonstrated this at the end of the visit to remind them. After each visit, the data were downloaded onto a computer, analysed using TM-2430-13 Doctor-Pro Software and checked for accuracy by the author. The first 3 readings obtained were discarded. Other values were rejected if the measurements of DBP and SBP were too close, DBP was ≥ 105 mm Hg or the heart rate (HR) or SBP showed a sudden sharp increase. If it was considered by the researcher that there were not enough accurate measurements (at least 22 awake measurements and 6 sleep measurements), the participant was requested to wear the monitor again for 24 h. SBP, DBP and HR values and the number of accurate readings were recorded for the day-time, night-time and 24 h readings. After fitting the monitor, the researcher recorded details of where and when (date and time) the monitor could be collected and the contact information of the participant. Monitors were usually collected within a day or two of the measurements being taken. A courier was booked and the monitor was returned directly to the researcher at KCL.

Figure 2.9 Fitting of the ABP monitor

2.3 Collection and handling of blood samples

2.3.1 Venepuncture

Screening blood samples were obtained following a 3 h fast. For the vitamin D_2/D_3 study, participants were not required to fast for any of the blood samples once on the study. In the DRISK study, participants were required to fast (nothing but water) from 10 pm the previous evening for each visit, once on the study. The participant was made aware of how much blood would be taken and explained the procedure clearly, prior to taking the sample. They were given a choice as to which arm they would prefer blood to be taken from. If the participant mentioned feeling faint on previous blood taking occasions, they were given the option of lying down on the couch. Otherwise, they were asked to sit in a phlebotomy chair and rest their arm on a pillow on top of the chair arm. All phlebotomy was performed by a trained phlebotomist and careful hand washing was performed before each procedure. Initially, a tourniquet was placed around the upper section of the arm and pulled tight. An antiseptic swab was then used to clean the site where blood was to be taken from. The vacutainer method (Becton-Dickinson) was used for all sample collections. This involved screwing the needle into the disposable vacutainer holder, unsheathing it, and inserting it into the brachial vein with minimal pressure. The vacutainer was then attached and the

tourniquet released. Details of the vacutainers used can be found in the blood handling protocols detailed in **Appendix 6** (D_2/D_3 study) and **Appendix 7** (DRISK study). They were collected in the following order according to the Becton-Dickinson Vacutainer Order of Draw for Multiple Tube Collections: Sodium Citrate Tube, Serum Separator Tube, EDTA Tube and Fluoride Oxalate Tube. On removal of the needle, a cotton wool ball was placed at the site and the participant asked to apply pressure to it until the bleeding had stopped. The needle was disposed of in a sharps bin and a plaster applied to the site if the participant had no known allergies to plasters on enquiry by the phlebotomist.

2.3.2 Sample processing

After collection of the blood, the vacutainers were inverted a number of times according to the Becton-Dickinson Vacutainer Order of Draw for Multiple Tube Collections. They were placed in ice or kept at room temperature before centrifugation according to the processing requirements for specific samples. Precise details of sample handling are listed in **Appendix 6** (D_2/D_3 study) and **Appendix 7** (DRISK study).

2.3.3 Coding and storage of samples

All vacutainers were labelled before each appointment with subject ID, gender and date of birth using permanent marker. Eppendorf tubes (2.0 mL Everyday SC Microtube Skirt NS, Cat. No. CP5518) were labelled prior to each visit using printed stickers resistant to -80˚C freezing (Tubees® HI-LOW Labels for laser printers, 25x13 mm, Cat. No. RTP/101). The format of the labels is shown in **Table 2.4**.

Table 2.4 Standard label format for eppendorf tubes. VDS, D_2/D_3 study); DRS, DRISK study. Visits were labelled A=screening, B=baseline, C=visit 1 etc.

PI	Study Name	ID	Visit
TS	VDS or DRS	001	A
	Test		
	PTH		
	Date		
	13.4.2011		

Croyovial boxes with 100 wells were used to store the samples. Due to the small number of participants, two or three different outcomes were stored in one box. Each box was clearly labelled with the PI's name, the author's name, King's College London (KCL), the study name, the type of samples inside and the date of initial sample collection. As soon as possible and no more than 2 h after collection, the plasma/serum samples were stored in a -80˚C freezer at KCL (if screening samples, or D_2/D_3 study samples), or at the clinical research facility (CRF) at St Thomas' Hospital (all DRISK study samples).

2.3.4 Transport of blood samples

Screening visit samples were analysed fresh by the CPA (Clinical Pathology Accredited) laboratory of the Department of Clinical Biochemistry (King's College Hospital (KCH), London) under the supervision of Roy Sherwood and Tracy Dew. They were placed in a clear plastic sealable bag per subject and then into a sealed padded envelope with biohazardous tape placed around it. An accredited medical courier (City Sprint PLC) collected the samples from the 4th Floor main reception, Franklin-Wilkins Building at KCL on the day of collection and transported them to KCH.

Samples collected during the study were packed and transported according to the UN3733 guidelines for shipment of biological samples. These guidelines specified that the samples be stored in a primary water-tight inner receptacle (Eppendorf tubes), have enough absorbent material (paper towels) between the primary and secondary receptacle to absorb the entire contents of the primary receptacle, be packaged inside a secondary watertight inner receptacle (the cryovial storage box) with sturdy outer

packaging (a polystyrene box filled with dry ice). The polystyrene box was then placed in a snug fitting cardboard box which was sealed using tape. The cardboard box had the following labels: contact details of the sender and receiver, a UN3373 label and a Class 9 danger label UN1845 which had the weight of dry ice inside the box written on it.

2.4 Analytical methods

Unless stated otherwise below, all plasma and serum samples were sent to the CPA accredited laboratory at the Department of Clinical Biochemistry at KCH.

2.4.1 Glucose

The ADVIA 2400 and reagents were supplied by Siemens Healthcare Diagnostics Ltd (Newton House, Sir William Siemens Square, Frimley, Camberley, Surrey, GU16 8QD). Blood was collected into a 4 mL fluoride oxalate tube (Becton-Dickinson, Cat no. 368920) and centrifuged at 4˚C at 1300 g for 15 min to separate the plasma. Initially, glucose present in the plasma was phosphorylated by adenosine triphophosphate (ATP) via the action of hexokinase. This produced glucose-6-phosphate which was then converted to 6-phosphogluconate in the presence of oxidized nicotinamide adenine dinucleotide (NAD), by glucose-6-phosphate dehydrogenase. The reduction of NAD+ to NADH resulted in an increase in absorbance at 340 nm which was proportional to the glucose concentration in the sample. The inter-assay precisions (% coefficient of variation, CV) were 1.6% and 1.2% and the intra-assay CVs were 0.6% and 0.5% for low and high QCs, respectively.

2.4.2 Haematology

A full blood count was performed at screening to confirm normal haematology. Blood was collected into a 2 mL vacutainer containing Ethylenediaminetetraacetic acid (EDTA) (Becton-Dickinson, Cat no. 367836) and kept at room temperature before being sent to KCH. The Siemans Advia 2120 and reagents were supplied by Siemans Medical Solutions Diagnostics Limited. Reference ranges for each of the haematology outcomes are displayed in **Table 2.5**. If values were outside of the ranges, they were checked by a clinician who would sign the subject off as suitable for participation in the study,

recommend that another blood sample was taken and tested, or advise that the subject was not suitable for participation in the study.

Table 2.5 Normal reference ranges for haematology outcomes

Haematology Outcome	Males	Females
WBC (10^9/L)	4.0 – 11.0	
RBC (10^{12}/L)	4.5 – 5.8	3.8 – 5.8
HB (g/dl)	13.0 – 16.5	11.5 – 15.5
MCV (fL)	77 - 95	
MCH (pg)	25 – 34	
MCHC (g/dL)	33.0 – 37.0	
PLT (10^9/L)	150 – 450	
Neutrophils (10^9/L)	2.2 – 6.3	
Lymphocytes (10^9/L)	1.3 – 4.0	
Monocytes (10^9/L)	0.2 – 1.0	
Eosinophils (10^9/L)	0 – 0.4	
Basophils (10^9/L)	0 – 0.1	

WBC, white blood cells; RBC, red blood cells; HB, haemoglobin; MCV, mean corpuscular volume; MCH, mean corpuscular haemoglobin; MCHC, mean corpuscular haemoglobin concentration; PLT, platelet count.

2.4.3 Liver function

Normal liver function was confirmed for eligible subjects at screening in both the D_2/D_3 and DRISK study using a number of colourimetric assays. Blood was collected into a 5 mL gel separator serum tube (Becton-Dickinson, Cat no. 367954) and, after leaving to stand at room temperature for 30 min, centrifuged at 1300 g for 15 min at 4˚C. Serum was dispatched to KCH on the same day, and spare serum stored at -80˚C. The ADVIA 2400 Clinical Chemistry System and reagents were supplied by Siemens Healthcare Diagnostics Ltd. Total protein was measured using a Biuret reagent (an alkaline solution of copper ions) that reacts with proteins and polypeptides containing at least two peptide bonds to produce a violet coloured complex, the absorbance of the complex at 540/660 nm is directly proportional to the concentration of protein in the sample. Albumin was measured using the dye Bromocresol green (BCG) which preferentially binds serum albumin to cause a shift in the absorption spectrum. The increase in absorbance measured at 596 nm is directly proportional to the albumin

concentration. Alkaline Phosphatase (ALP) was measured by the addition of para-nitrophenyl phosphate (PNPP) which is hydrolysed by ALP to form free phosphate and para-nitrophenol, a yellow coloured dye. The rate of increase in absorbance measured at 410 nm is directly proportional to ALP activity in the sample. The addition of the Siemen's Healthcare Diagnostics reagents to Bilirubin led to its oxidation and consequently a decrease in its concentration. Bilirubin concentrations were measured at 451 nm using the change in absorbance over a 5 min reaction period. GGT, on addition of the Siemen's reagents, catalyses a reaction which liberates 4-nitroaniline. The increase in absorbance of 4-nitroaniline at 410 nm is directly proportional to the activity of GGT in the sample. Reference ranges for each of the liver function outcomes are displayed in **Table 2.6**. If values were outside of the ranges, they were checked by a clinician who would sign the subject off as suitable for participation in the study, recommend that another blood sample was taken and tested, or advise that the subject was not suitable for participation in the study.

Table 2.6 Normal reference ranges for liver function outcomes

Liver Function Outcome	Males	Females
Total protein (g/L)	60 - 80	
Albumin (g/L)	35 - 50	
Total Bilirubin (µmol/L)	3 - 20	
ALP (IU/L)	30 - 130	45 - 130
AST (IU/L)	10 - 50	3 - 35
GGT (IU/L)	1 - 55	
Globulin (g/L)	25 - 35	

ALP, alkaline phosphatase; AST, aspartate transaminase; GGT, gamma glutamyltranspeptidase

2.4.4 Lipids

Blood samples were collected into 5 mL (Becton-Dickinson, Cat no. 367954) or 8.5 mL (Becton-Dickinson, Cat no. 367958) gel separator tubes, left to stand at room temperature for 30 min, centrifuged at 1300 g for 15 min, and the serum separated. The ADVIA 2400 Clinical Chemistry System (Siemens Healthcare Diagnostics Ltd) was used to run colourimetric assays to measure TC, HDL-C and triglycerides. Screening visit samples were sent to the laboratory at KCH on the day of collection for

determination within 4 days of collection. The other samples were stored at -80˚C and analysed in batches once the study was finished. LDL-C was calculated by the Friedwald formula:

LDL cholesterol = Total cholesterol - HDL cholesterol - (Triglyceride/2.2)

(The calculation becomes invalid if triglyceride is > 4.5)

2.4.4.1 Total cholesterol

Serum TC was determined using an enzymatic method in which cholesterol esterase completely hydrolyses cholesterol esters to free cholesterol, which is in turn oxidised by cholesterol oxidase, generating hydrogen peroxide. The hydrogen peroxide formed combines with 4-aminophenazone and a phenol to form a red quinone amine dye. The absorbance of this was measured at 505/694 nm with the increase in dye absorbance being directly proportional to the concentration of cholesterol in the sample. The cholesterol reagent was supplied by Siemens Healthcare Diagnostics Ltd. The inter-assay CVs were 1.1%, 1.5% and 1.0% and the intra-assay CVs were 0.6%, 0.6% and 0.6% for low, medium and high QCs, respectively.

2.4.4.2 HDL cholesterol

HDL-C was measured in serum using a two-step automated procedure using reagents supplied by Siemens Healthcare Diagnostics Ltd. In the first step, cholesterol esterase and cholesterol oxidase react to remove non-HDL-C from the sample. The hydrogen peroxide produced is then removed by the enzyme catalase. In stage 2, detergent is added to allow HDL-C to react with the enzyme system. Sodium azide inhibits the reaction of the hydrogen peroxide formed with catalase. The hydrogen peroxide then reacts with 4-aminoantipyrine to produce a quinoneimine pigment measured at 596 nm. The resulting colour is proportional to the concentration of HDL-C. The inter-assay CVs were 2.2%, 2.1% and 2.5% and the intra-assay CVs were 1.1%, 1.4% and 1.5% for low, medium and high QCs, respectively.

2.4.4.3 Triglycerides

Reagents were supplied by Siemens Healthcare Diagnostics Ltd. Triglycerides were first converted to glycerol and free fatty acids by the addition of lipase. The glycerol was

then converted to glycerol-3-phosphate by glycerol kinase and glycerol-3 phosphate was subsequently converted to hydrogen peroxide by glycerol-3-phosphate-oxidase. The hydrogen peroxide produced reacted with a chromophore under the catalytic influence of peroxidise and the absorbance of the complex, which is proportional to the triglyceride concentration, was measured as an endpoint reaction at 505/694 nm. The inter-assay CVs were 2.5% and 1.5% and the intra-assay precisions were 0.6% and 0.5% for low and high QCs, respectively.

2.4.5 Calcium

Calcium was measured on the ADVIA 2400 Clinical Chemistry System (Siemens Healthcare Diagnostics Ltd) using a colourimetric assay. Blood samples were collected into 5 mL (Becton-Dickinson, Cat no. 367954) or 8.5 mL gel separator tubes (Becton-Dickinson, Cat no. 367958), left to stand at room temperature for 30 min, and centrifuged at 1300 g for 15 min at 4˚C. The sample was sent at room temperature on the day of collection to KCH for analysis. Calcium reagents were supplied by Siemens Healthcare Diagnostics Ltd. Reagent 1 (ethanolamine buffer) provided an alkaline medium in which calcium was able to react with cresolpthalein complex one (from reagent 2) to form a violet coloured chromophore which was monitored at its absorbance maximum of 545 nm. The absorbance was proportional to the sample's calcium concentration. The inter-assay CVs were 2.3%, 2.3% and 1.9% and the intra-assay CVs were 0.8%, 2.0% and 0.7% for low, medium and high QCs, respectively.

2.4.6 Vitamin D

There are several available methods for measuring 25-OH-D metabolites (**Table 2.7)**. The two main types of methods used are competitive immunoassays [radioimmunoassay (RIA), enzyme-linked immunoassay (EIA) and chemiluminescence immunoassay (CIA)] and those which involve a chromatographic separation, followed by detection of the analyte without the use of antibodies [High-Pressure Liquid Chromatography with UV Detector (HPLC-UV) and liquid chromatography tandem mass spectrometry (LC-MS/MS)]. Inter-laboratory discrepancies have been reported between the results of these different assays (Binkley *et al*. 2004). Standardisation of methods is necessary to bring laboratories into alignment and ensure accuracy in order to allow for comparison of studies and the development of evidence-based guidelines.

The National Institute of Standards and Technology (NIST) has developed a Standard Reference Material, SRM 972 which consists of four blood serum sample pools with varying levels of 25-OH-D and certified values for 25-OH-D_2, 25-OH-D_3, and 3-epi-25-OH-D_3 (Phinney 2008). This can be used as a reproducible point of comparison of accuracy between methods and to validate new methods as they are developed (Phinney 2008). DEQAS (International Vitamin D External Quality Assessment Scheme) is also available for laboratories to participate in to ensure the analytical reliability of 25-OH-D assays. Human unprocessed serum is distributed quarterly to participating laboratories and a Proficiency Certificate is issued to those whose returned results meet the performance targets set by the DEQAS Advisory Panel (DEQAS 2014). Dependant on what the purpose of the 25-OH-D measurement is, certain methods may be more appropriate to use than others. For example, only the HPLC-UV and LC-MS/MS methods are able to measure 25-OH-D_2 and 25-OH-D_3 separately and equally well (de la Hunty *et al.* 2010), whereas generally immunoassays, although able to detect both metabolites, may not be able to accurately detect the proportion of 25-OH-D_2 (Carter *et al.* 2007). In terms of precision, all the methods are similar (de la Hunty *et al.* 2010), apart from LC-MS/MS which is better. For diagnosis purposes, all the methods described above are able to measure 25-OH-D concentrations <25 nmol/L which represents deficiency, although LC-MS/MS has the highest sensitivity. LC-MS/MS has greater accuracy compared to competitive immunoassays; compared to the target value of the NIST SRM 972, LC-MS/MS results showed a smaller mean deviation (% bias) of +1.9% compared to between -14.7% and -11.6% for two different CIAs (Moon *et al*. 2012). The advantage of the immunoassays is that they are more convenient and have high throughput capabilities. HPLC-UV and LC-MS/MS both require an initial sample extraction stage which, although useful as it removes interfering substances, is time-consuming and labour-intensive, meaning that less samples can be processed in a certain time period (de la Hunty *et al.* 2010). Furthermore, although semi-automated or automated procedures have been developed for HPLC-UV and LC-MS/MS, immunoassays have much quicker run times, particularly if an automated procedure is used (Wallace *et al.* 2010). The cost of the assay is another factor to take into account when deciding which assay to use. LC-MS/MS and HPLC-UV are expensive (£25-£27 per sample) due to the high cost of equipment and need for highly skilled staff, compared with immunoassays for which although reagent costs may be higher, the cost per

sample is around £10-£17 (Wallace *et al.* 2010). Another factor to consider is the potential interference of 3-epi-25-OH-D_3. In October 2011 a DEQAS survey assessed the ability of immunoassays, HPLC and LC-MS/MS to distinguish between epi-25-OH-D_3 and 25-OH-D_3 by giving laboratories two matched samples; one containing only 25-OH-D_3 and the other additionally containing 50 nmol/L epi-25-OH-D_3. The majority of immunoassays including the ADVIA Centaur CIA do not recognise 3-epi-25-OH-D_3, but HPLC and LC-MS/MS detected both epi-25-OH-D_3 and 25-OH-D_3. With LC-MS/MS, many laboratories are unable to resolve 3-epi-25-OH-D_3 from 25-OH-D_3 as most methods cannot differentiate between compounds which have an identical elemental composition but different structure, and this can result in an overestimation of the true 25-OH-D_3 concentration (Bailey *et al*. 2013). However, in adults, the percentage contribution of 3-epi-25-OH-D_3 to total 25-OH-D_3 appears to be small (Bailey *et al*. 2013). Overall, LC-MS/MS is considered to be the best assay for detection and quantification of serum 25-OH-D, although if cost is an issue and it is not necessary to obtain the proportions of 25-OH-D_2 and 25-OH-D_3 that are contributing to total 25-OH-D, it may be preferable to use an immunoassay (de la Hunty *et al.* 2010).

For both the D_2/D_3 and DRISK studies, it was necessary to be able to measure 25-OH-D_2 and 25-OH-D_3 separately to measure the responses to D_2 and D_3 supplementation and to check that there was no increase in 25-OH-D_3 as a consequence of UVB exposure. It was decided to use LC-MS/MS for the duration of both studies as it is able to measure the two 25-OH-D metabolites separately with high specificity and high precision. The present study used an improved method which uses a smaller sample (0.1 mL) in ultra high pressure liquid chromatography tandem mass spectrometry (UPLC-MS/MS) (Ding *et al.* 2010) compared with conventional LC-MS/MS assays which use 0.5-1.0 mL. In order to reduce run-to-run variability, samples were batched so that the analyses for the same individual were completed in the same run. The uHPLC method was developed by HFL Sport Science (Fordham, Cambridgeshire) which are now part of the Laboratory of the Government Chemist. For screening in the DRISK study, and in the CRESSIDA and MARINA studies, plasma total 25-OH-D was determined by a chemiluminescent immunoassay as detailed below because of the lower cost and the speed of turnaround of samples to enable a decision to be made to exclude participants in the DRISK study who had high 25-OH-D concentrations at screening.

Table 2.7 Summary of methods for measurement of 25-OH-D adapted from (de la Hunty *et al.* 2010) and (Wallace *et al.* 2010).

	RIA	CIA	HPLC-UV	LC-MS/MS	uHPLC MS/MS
Ability to measure $25OHD_2$ and $25OHD_3$ separately	Poor	Poor	Excellent	Excellent	Excellent
Ability to measure $25OHD_3$ and $25OHD_2$ equally well	Acceptable	Acceptable	Excellent	Excellent	Excellent
Precision	Acceptable	Good	Good	Good	Excellent
Cost per sample (approx)	£17	£10	£15	£25	£27

RIA, Radioimmunoassay; CIA, Chemiluminescent immunoassay; HPLC-UV, High-Pressure Liquid Chromatography with UV Detector; LC-MS/MS, Liquid chromatography-tandem mass spectrometry.

2.4.6.1 Measurement of 25-OH-D metabolites by UPLC-MS/MS

25-OH-D_2 and 25-OH-D_3 were measured using UPLC-MS/MS. Blood samples were collected into 5 mL gel separator tubes (Becton-Dickinson, Cat no. 367954), left to stand at room temperature for 30 min, and centrifuged at 1300 g for 15 min at 4˚C. The serum was pipetted into eppendorfs and stored in the -80˚C freezer until the end of the study. It was then sent along with all the other vitamin D samples from the rest of the study to HFL Sport Science (Fordham, Cambridgeshire) for analysis. Internal standards used were hexadeuterated 25-OH-D_2 and 25-OH-D_3 (25-hydroxyvitamin D_2-(26,26,26,27,27,27-D6) and 25-hydroxyvitamin D_3-(26,26,26,27,27,27-D6)). These were spiked into 100 µl of sample along with a protein precipitation reagent, mixed, and placed in a centrifuge in order to precipitate out all of the protein before being analysed by UPLC-MS/MS. The LC stage uses a reverse phase chromatography column (Waters C_{18} Acquity column 1.8 µm 2.1 x 50 mm) with a flow rate of 0.6 mL/min. which flushes away hydrophilic interfering compounds. Hydrophobic compounds, including 25-OH-D in the sample and the internal standards, stick to alkyl chains on the silica based particles in the column. Organic solvent is gradually introduced to take

away these compounds. The remaining sample then travels to a mass spectrometer (MS) analytical column which ionises the compounds in a vacuum into positively charged ions. As there are many steroids with a similar ratio, the compounds are then forced through a filter filled with inert gas to fragment the ions; the way in which fragmentation occurs is specific to the compound. Subsequently, the ion fragments are accelerated so that they all have the same kinetic energy. They are then deflected by a magnetic charge according to their mass:charge ratio (M/E), with the lighter and more charged ions being deflected more. The MS counts the number of ion fragments deflected for each mass:charge ratio. The output has peaks for 25-OH-D_2, 25-OH-D_3 and the two deuterated internet standards (**Figure 2.10**). The assay was fully validated, including the elements of dynamic range, sensitivity, selectivity, matrix effects, carry over, precision and accuracy. Each sample batch contained quality control (QC) samples at three concentrations (low, medium and high) with each QC level analysed in duplicate. Batch acceptance criteria were that no more than two QC samples were allowed to deviate by more than 15% from their target value within each batch, with those two failed QCs having to be at different concentrations. The inter-assay precisions (%CV) for low, medium and high QCs for 25-OH-D_2 were 5.9%, 6.7% and 7.3%, and accuracies (%RE) were 1.2%, 2.3% and 0.5% respectively. The intra-assay precisions for low, medium and high QCs for 25-OH-D_3 were 5.4%, 7.5% and 8.2%, and accuracies were 6.6%, 5.6% and 3.4% respectively. The assay was validated over the range 1 to 150 ng/mL for 25-OH-D_2 and 3 to 150 ng/mL for 25-OH-D_3. The lowest detectable limit for 25-OH-D_2 was 2.4 nmol/L.

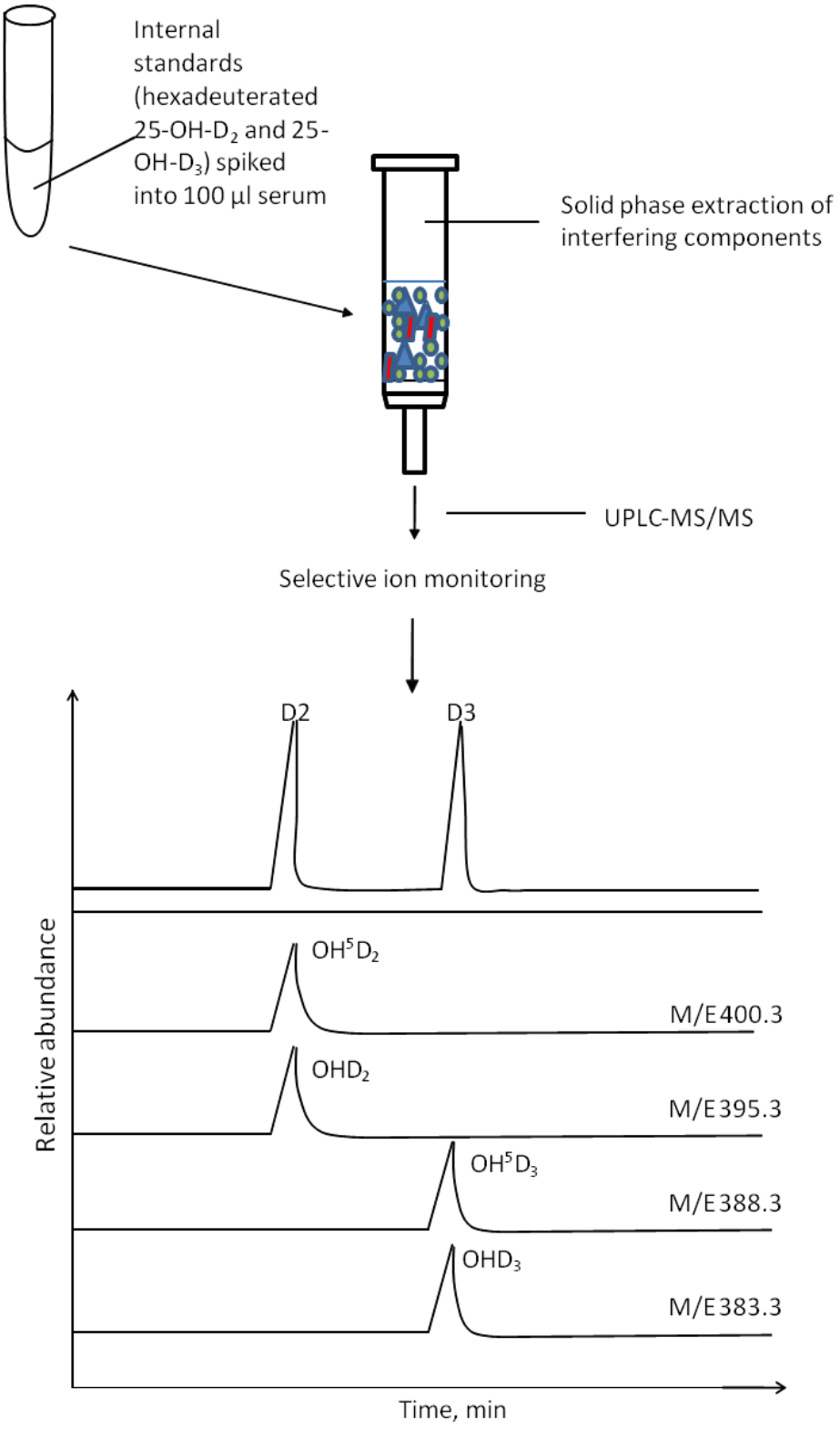


Figure 2.10 Vitamin D assay (UPLC-MS/MS)

2.4.6.2 Measurement of 25-OH-D by competitive chemiluminescence immunoassay

Total 25-OH-D was measured in the DRISK study at screening and in the MARINA and CRESSIDA studies using a chemiluminescent immunoassay at the Department of Clinical Biochemistry at KCH (KingsPath). This laboratory participates in DEQAS (DEQAS 2014), and has been issued a Proficiency Certificate (**Appendix 8**). Blood samples were collected into 5 mL gel separator tubes (Becton-Dickinson, Cat no. 367954), left to stand at room temperature for 30 min, and centrifuged at 1300 g for 15 min at 4˚C. The serum was sent at room temperature on the day of collection to KCH for analysis. Vitamin D reagents were supplied by Siemens Healthcare Diagnostics Ltd and the assay was run on the Siemens Advia Centaur analyser. The serum was added to a cuvette and treated to remove 25-OH-D from binding proteins. The resulting mixture was then incubated with an antibody to 25-OH-D bound to acridinium ester. A vitamin D analogue (labelled with fluorescein) was added together with an antibody to fluorescein bound to paramagnetic particles (PMP). 25-OH-D in the sample competes with the vitamin D analogue for binding to the 25-OH-D antibody, such that when there is a higher concentration of 25-OH-D present in the sample, less of the analogue binds. A magnetic field was applied to the reaction mixture causing the solid-phase PMP (which is bound to the antibody-antigen complex) to be held at the side of the reaction cuvette while the liquid phase was aspirated. After a couple of washes of the cuvette contents with a phosphate-buffered saline, acid reagent (containing hydrogen peroxide) was added to the cuvette to begin the light-emission reaction with the acridinium ester. The cuvette was then moved to the luminometer where base reagent was added to enhance the light reaction. The light intensity was measured immediately and converted to 'relative light units' (RLU). The RLU value has an inverse relationship with 25-OH-D concentration (the higher the RLU value, the lower the 25-OH-D concentration). The inter-assay CVs were 11.1%, 9.8% and 4.8%, and the intra-assay CVs were 7.0%, 5.8% and 2.7% for low, medium and high QCs, respectively. The assay was validated for serum concentrations up to 374 nmol/L. The lowest detectable limit was 8.0 nmol/L.

2.4.7 Parathyroid hormone (PTH)

PTH was measured on a Siemens Centaur by immunoassay, and PTHi reagent was supplied by Siemens Healthcare Diagnostics Ltd. Blood samples were collected into 2 mL (Becton-Dickinson, Cat no. 367836) or 4 mL EDTA tubes (Becton-Dickinson, Cat no. 367839), stored in ice, and centrifuged at 1300 g for 15 min at 4˚C before being stored within 2 h of collection at -80˚C. Plasma samples were sent at the end of the study to KCH for analysis with all other PTH samples collected during the study. The plasma sample was incubated with two PTH-specific antibodies. The first was labelled with acridinium ester (AE) and the second was a biotinylated polyclonal goat anti-human PTH antibody. Streptavidin (biotin-binding protein) in the Solid Phase is covalently coupled to paramagnetic particles (PMP). PTH forms a 'sandwich' between the two antibodies. After the incubation period a magnetic field is applied to the reaction mixture causing the solid-phase PMP (including the 'sandwich') to be held at the side of the reaction cuvette while the liquid phase is aspirated. The cuvette contents are washed with deionised water which is then aspirated, again with the magnetic field applied. Acid reagent (containing hydrogen peroxide) is added to the cuvette to begin the light-emission reaction with the acridinium ester. The cuvette is moved to the luminometer at which point base reagent is added to enhance the light reaction. The light intensity is measured immediately and converted to 'relative light units' (RLU). The RLU value has a direct relationship with PTH concentration (i.e. the higher the RLU value, the higher the PTH concentration). The inter-assay CVs were 5.2%, 3.4% and 3.5% and the intra-assay precisions were 4.3%, 23.7% and 91.1%, for low, medium and high QCs, respectively. The assay was validated for serum concentrations up to 374 nmol/L. The limit of detection was 8.0 nmol/L.

2.4.8 Renin

Renin was measured by chemiluminescence immunoassay in the DRISK study at baseline, mid-point and end-point on a Liaison chemiluminescence analyser (Diasorin, Charles House, Toutley Road, Wokingham, Berkshire) using the Diasorin Liaison direct renin reagent. Blood samples were collected into 4 mL EDTA tubes (Becton-Dickinson, Cat no. 367839) and centrifuged at 1300 g for 15 min at room temperature before being stored at -80˚C. Plasma samples were sent at the end of the study to KCH for analysis. The assay utilises PMP coated with a mouse monoclonal antibody that

recognises both renin and prorenin and another monoclonal antibody specific for renin which is linked to an isoluminol derivative (isoluminol-antibody conjugate). During the incubation, renin and prorenin in samples bind to the solid phase monoclonal antibody. Subsequently the antibody conjugate reacts with renin already bound to the solid phase to form a soluble sandwich complex. Following incubation, the unbound material is removed by a wash cycle. The wells containing the washed magnetic particles are transported into the system luminometer which initiates a flash chemiluminescence reaction by the addition of hydrogen peroxide and an alkaline solution to oxidise the isoluminol derivative. The light produced is quantified by the luminometer and expressed as relative light units (RLU). The amount of bound labelled antibody is directly proportional to the concentration of renin in the sample. The inter-assay CVs were 10.0%, 12.4% and 5.7% and the intra-assay CVs were 3.7%, 2.8% and 2.0% for low, medium and high QCs, respectively.

2.4.9 High sensitivity C-reactive protein

HsCRP was measured using Immuno Turbidimetry. Blood samples were collected into 8.5 mL gel separator tubes (Becton-Dickinson, Cat no. 367958), left to stand at room temperature for 30 min, and centrifuged at 1300 g for 15 min at room temperature before being stored at -80 ˚C. HsCRP was analysed using an anti-CRP antibody sensitized to latex particles (P.Z. Cormay, Lubin, Poland) which coats latex particle surfaces and then reacts with CRP in the sample resulting in visible agglutination. The degree of agglutination was detected by turbidimetry at 572 nm as a reduction in the intensity of transmitted light on an ADIVA 2400 analyser (Sieman's Healthcare Diagnostics, Frimley, Surrey, UK). The inter-assay CVs were 6.97, 3.34 and 1.23% and the intra-assay CVs were 5.74, 1.99 and 1.16% for low, medium and high QCs.

2.4.10 MMP-9

MMP-9 was measured using a Quantikine MMP-9 enzyme-linked immunosorbent assay (ELISA) kit distributed by R & D Systems Europe (19 Barton Lane, Abingdon Science Park, Abingdon, Oxon, OX14 3NB) on serum. Blood samples were collected into 8.5 mL gel separator tubes (Becton-Dickinson, Cat no. 367958), left to stand at room temperature for 30 min, and centrifuged at 1300 g for 15 min at room temperature before being stored at -80˚C. Serum samples were sent at the end of the

study to KCH for analysis. The assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for MMP-9, pre-coated onto a microplate is provided in the kit. Standards and samples were pipetted into the wells and any MMP-9 present was bound by the immobilsed antibody. After washing away any unbound substances, an enzyme-linked monoclonal antibody specific for MMP-9 was added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution was added to the wells and colour developed in proportion to the amount of MMP-9 bound in the initial step. The colour development was stopped and the intensity of the colour measured. The inter-assay CVs were 7.9%, 7.8 and 6.9% and the intra-assay CVs were 2%, 1.9% and 2.9% for low, medium and high QCs, respectively.

2.4.11 C-peptide

C-peptide was measured by immunoassay on the Siemens Immulite 2000 analyser in a solid-phase, two-site chemiluminescent immunometric assay. Reagents were supplied by Siemens Healthcare Diagnostics Ltd (Newton House, Surrey GU16 8QD). Blood samples were collected into 8.5 mL gel separator tubes (Becton-Dickinson, Cat no. 367958), left to stand at room temperature for 30 min, and centrifuged at 1300 g for 15 min at room temperature before being stored at -80˚C. Serum samples were sent at the end of the study to KCH for analysis. The solid phase (bead) was coated with a monoclonal anti-C-peptide antibody. The liquid phase consisted of alkaline phosphatase (ALP) conjugated to monoclonal anti-C-peptide antibody in buffer. The sample and reagent were incubated together with the coated bead for 30 min. During this time, C-peptide in the sample forms an antibody sandwich complex with monoclonal anti-C-peptide antibody in the reagent. Unbound sample and enzyme conjugate were then removed by centrifugal washes. Finally, a chemiluminescent ALP substrate was added to the reaction tube containing the bead. ALP bound to the antibody complex dephosphorylates the substrate and this releases light which is measured by a luminometer. The signal generated is in proportion to the concentration of C-peptide. The inter-assay precisions CVs were 2.9%, 2.5 and 1.6%, and the intra-assay CVs were 3.6%, 1.2% and 3.0% for low, medium and high QCs, respectively.

2.4.12 Haemostatic assays – Fibrinogen and Factor VII

Fibrinogen and FVII are involved in a coagulation cascade that helps to arrest bleeding in response to vascular injury, but when disrupted can lead to thrombosis and bleeding (Borissoff *et al.* 2011). Initially, following vascular injury, tissue factor is exposed to the blood and this triggers a coagulation cascade beginning with the tissue factor (extrinsic) pathway shown in **Figure 2.11**. When thrombin is generated, an intrinsic pathway can be triggered and the two pathways converge (Davie *et al.* 1991). Here, only the extrinsic pathway will be discussed as it involves FVII and fibrinogen which are relevant to this thesis. Tissue factor has a high affinity for FVII, and the two form a catalytic complex in the presence of calcium ions which facilitates the conversion of FVII to $FVII_a$. Subsequently, the $FVII_a$-tissue factor complex converts factor X to factor Xa. These two reactions rely on tissue factor acting as a cofactor to accelerate the conversions. In order for prothrombin to be converted to thrombin, Factor Xa, in the presence of calcium ions and phospholipid, forms a complex with Factor Va known as prothrombinase. Once thrombin is formed, it converts fibrinogen to fibrin. After the formation of fibrin, the conversion of Factor XIII to Factor XIIIa by thrombin, in the presence of calcium ions is accelerated. Factor XIIIa enables incorporation of other plasma proteins into the fibrin clot. In addition to converting fibrinogen to fibrin, thrombin has an important role in the activation of platelets. It activates Factor XIII which induces side-by-side fibrin polymerisation to generate an insoluble, stable, fibrin clot, or thrombus in atherosclerosis. Additionally, it supports positive-feedback activation of the Factors V, VIII and XI, allowing for the amplification and propagation of coagulation. Further amounts of tissue factor, Factor V, Fibrinogen, and Factor XIII are introduced into the system on activation of platelets (Borissoff *et al.* 2011; Davie *et al.* 1991).

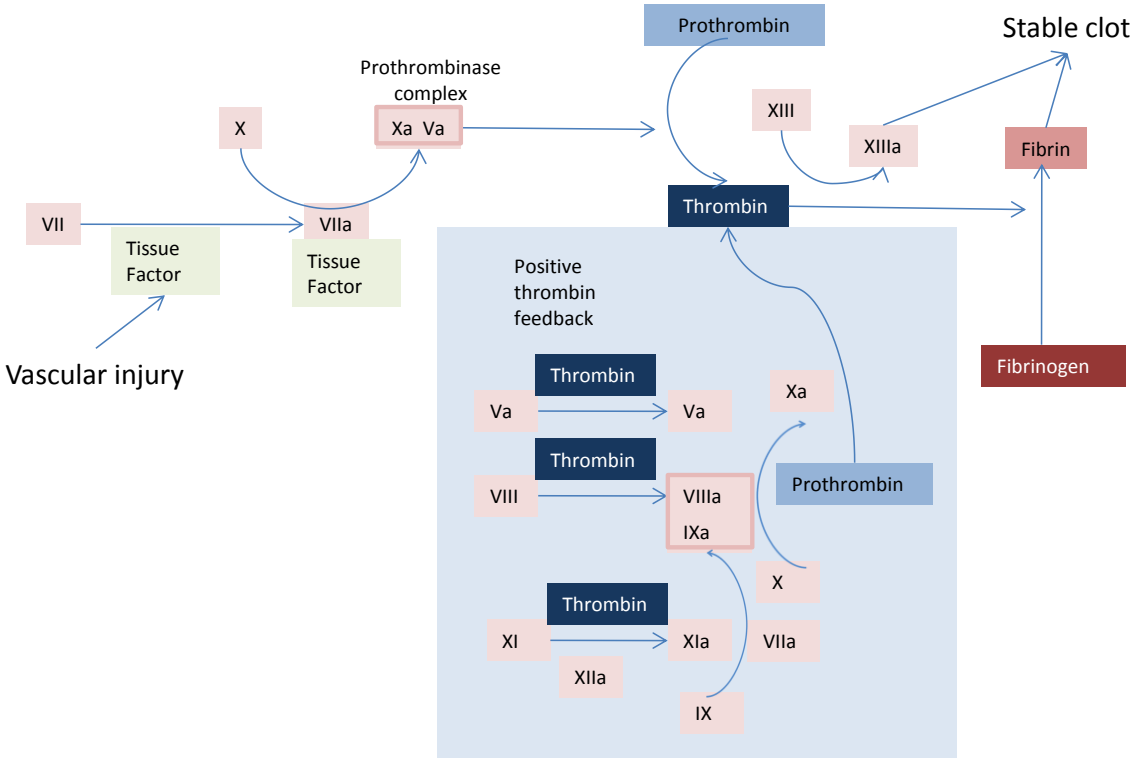


Figure 2.11 Tissue Factor Extrinsic Pathway taken from (Borissoff *et al.* 2011). Numbered Factors are abbreviated as IX, X, VII etc.

2.4.12.1 Factor VII

$FVII_c$ was measured on frozen citrated plasma samples that were rapidly thawed and brought to 37˚C to avoid cold activation. Blood samples were collected into 4.5 mL sodium citrate tubes (Becton-Dickinson, Cat no. 367958) and centrifuged at 1300 g for 15 min at room temperature before being stored at -80˚C within 2 h of collection. $FVII_c$ was measured using a one-stage bioassay on the STart® 4 Hemostasis Analyzer (coagulometer) (Diagnostica Stago UK Ltd, 2 Theale Lakes Business Park, Moulden Way, Theale, RG7 4GB). The sample plasma was thawed in a water bath set at 38˚C. It was then diluted 1:40 in Imidazole buffer (Diagen, Diagnostic Reagents Ltd, Thame, Oxon, OX9 3NY) before adding 50 µl of it to an equal volume of bovine FVII deficient plasma (prepared as described elsewhere (Miller *et al.* 1994)) in a cuvette well on the coagulometer. A metal bead was added, and the mixture warmed for 60 seconds at 38 ˚C. Coagulation was initiated by the addition of 100 µl of Calcium Rabbit Brain Thromboplastin (Diagen, Diagnostic Reagents Ltd, OX9 3NY) and the time taken for clot formation measured as when the metal ball stopped moving. $FVII_c$ was expressed as a percentage of the activity of a standard of known $FVII_c$ (standard human plasma SHP,

Diagnostica Stago UK Ltd, 2 Theale Lakes Business Park, RG7 4GB) by reference to its standard curve (**Figure 2.12**). This standard was calibrated against the WHO 2nd International Standard for FVII Concentrate (NIBSC code number: 10/252). The standard had a $FVII_c$ activity of 93%. It was reconstituted with 1.0 mL of distilled water and dilutions were prepared at 1:10, 1:20, 1:40 and 1:60 with imadazole buffer. The activity of the dilutions was recorded as 413.3%, 206.7%, 103.3% and 68.9% respectively (worked out as 5/4.5 x activity, to adjust for the fact that 0.5 mL in the vacutainer was sodium citrate and 4.5 mL plasma). Samples for each subject were analysed in the same run to avoid between-assay variation; the within-run CV was 2.0%.

Before measuring $FVII_c$ in the study samples, experimental work was carried out to optimise the method described above. The volume of plasma in each study sample eppendorf tube was approximately 1-1.5 mL. In order to work out how long to leave the sample in the water bath for and how long the $FVII_c$ in the sample was stable for once thawed, two samples of 1 mL and 1.5 mL plasma were taken from volunteers by a trained phlebotomist. Each sample was placed in the 38 ˚C water bath and temperature readings were taken every 30 seconds. The 1 mL and 1.5 mL samples took 5 min and 6.5 min respectively to reach 38˚C, and so it was decided to thaw the study samples for 6 min in order to prevent loss of activity by keeping the sample at 38˚C for too long. This experiment further demonstrated that thawing of the samples in the water bath led to a very quick rise in temperature of around 5 to 20˚C over a 30 sec period, so cold activation of $FVII_c$ at around 4-10˚C was unlikely. The stability of the plasma was also tested once it had reached 38˚C by running the assay every 5 min. Clotting times started to increase after 15 to 20 min so it was decided to only defrost 4 samples at once in order to run them all in duplicate on the coagulometer within 15 min. If the duplicate values were not within around 2-3% of each other, the sample was re-run. Another experiment was run to determine how long the deficient plasma remained stable for. The activity of a QC was measured every half an hour after thawing the deficient plasma to 38˚C, demonstrating that it remained stable for 2.5 – 3 h as the clotting time started to increase after this time. This experiment also demonstrated that the QCs, which were 1:40 diluted SHP, are stable when snap frozen in liquid nitrogen immediately after dilution, and placed in a -80˚C freezer.

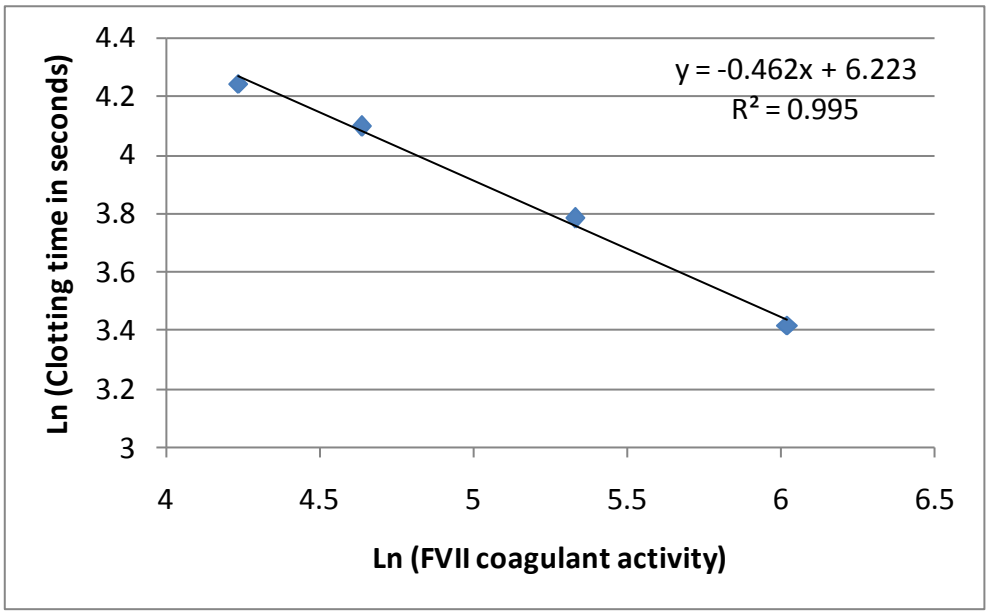


Figure 2.12 Standard curve showing clotting times for different dilutions of standard human plasma (SHP) for calibration purposes.

2.4.12.2 Fibrinogen

Fibrinogen was measured on frozen citrated plasma that was rapidly thawed and mixed. Blood samples were collected into 4.5 mL sodium citrate tubes (Becton-Dickinson, Cat no. 367958), centrifuged at 1300 g for 15 mins at room temperature before being stored at -80 ˚C within 2 h of collection. Fibrinogen concentration was determined using the von-Clauss thrombin-clotting method (A 1957) on the STart® 4 Hemostasis Analyzer (coagulometer) (Diagnostica Stago UK Ltd, 2 Theale Lakes Business Park, RG7 4GB). The sample plasma was diluted 1:20 in Owren-Koller buffer solution (Diagnostica Stago UK Ltd, 2 Theale Lakes Business Park, RG7 4GB) and 100 µl placed in a cuvette well in the coagulometer (Diagnostica Stago UK Ltd, 2 Theale Lakes Business Park, RG7 4GB), along with a metal bead. After warming in the well at 38 ˚C for 60 seconds, clot formation was initiated by the addition of 50 µl Fibri-Prest® Automate (Diagnostica Stago UK Ltd, 2 Theale Lakes Business Park, RG7 4GB). This mechanical clotting end point method measures the time it takes for the clot to form by measuring the time at which the metal ball stops vibrating. The fibrinogen concentration of the plasma was calculated by reference to a standard curve created by serial dilutions of a plasma of known fibrinogen concentration (Unicalibrator – Diagnostica Stago UK Ltd, 2 Theale Lakes Business Park, RG7 4GB) (**Figure 2.13**). Samples for each subject were analysed in the same run to avoid between-assay

variation; the within-run CV was 1.54%. Calculations for fibrinogen took into account the fact that 0.5 mL in the vacutainer was sodium citrate and 4.5 mL plasma.

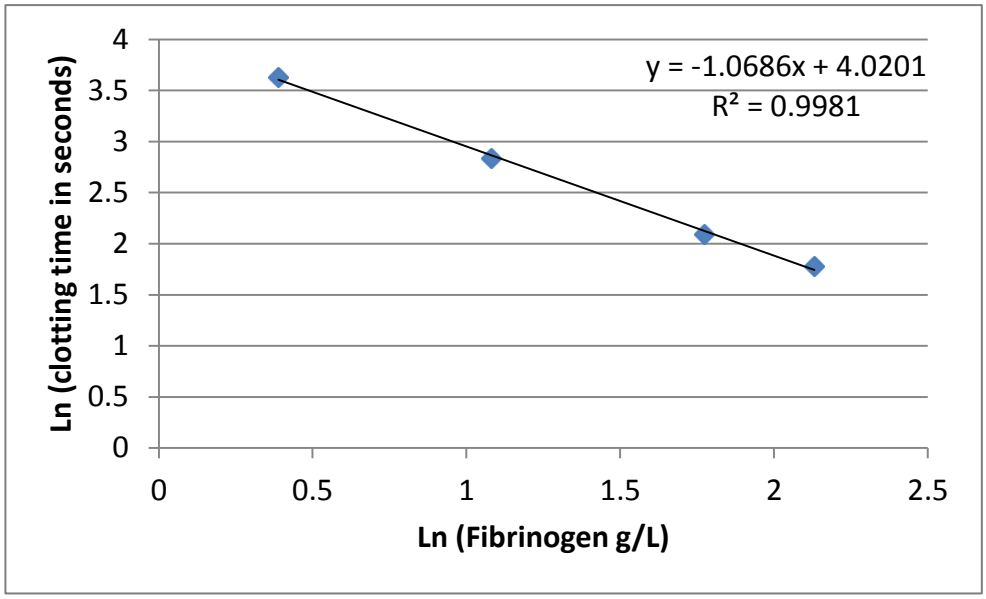


Figure 2.13 Standard curve showing clotting times for different dilutions of unicalibrator for calibration purposes.

2.4.13 Vitamin D binding protein (VDBP)

VDBP was measured in the DRISK study at baseline, mid-point and end-point using a quantitative sandwich enzyme immunoassay technique and a VDBP kit distributed by R&D Systems Europe, Ltd. (19 Barton Lane, Abingdon Science Park, Abingdon, OX14 3NB, United Kingdom). Blood samples were collected into 5 mL gel separator tubes (Becton-Dickinson, Cat no. 367954), left to stand at room temperature for 30 min, and centrifuged at 1300 g for 15 min at 4˚C. The serum was pipetted into eppendorfs and stored in the -80˚C freezer until the end of the study when it was sent to KCH for analysis. The assay kit contained a monoclonal antibody specific for VDBP already pre-coated onto a microplate. Standards and samples were pipetted into the wells and any VDBP present was bound by the immobilised antibody. After washing away any unbound substances, an enzyme-linked monoclonal antibody specific for VDBP was added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution was added to the wells. Colour developed in proportion to the amount of VDBP bound in the initial step. The colour development was stopped

and its intensity, proportional to the VDBP concentration, was measured. The inter-assay CVs were 5.1%, 6.0 and 7.4%, and the intra-assay CVs were 5.7%, 5.8% and 6.2% for low, medium and high QCs, respectively.

2.5 Dietary analysis

In this thesis the aim was to determine vitamin D and calcium intake from the diet. Different dietary assessment methods are available, but after consideration of the main strengths and limitations related to the required purpose of the studies in this thesis (**Table 2.8**), a food frequency questionnaire (FFQ) was chosen. A FFQ is suitable as, although it does not provide valid estimates of absolute intake in individuals, it ranks the intake of individuals relative to others in the population and can be used to identify changes in diet. It is also affordable, relatively quick and simple for the participant to complete and does not require an investigator to be present. Furthermore, it assesses diet over a longer period of time than other methods which allows for the capture of vitamin D intake, a nutrient present in only a limited number of foods such as oily fish, which may not be consumed regularly. However, the disadvantage of FFQs is that reported intake is limited to the foods contained in the food list which are unlikely to distinguish between, for example, fortified and non-fortified products. The CRESSIDA study (section 2.6.1), conducted by another study group, required a more detailed dietary analysis to assess energy and macronutrient intake as well as micronutrients, and so estimated 4-d food diaries were completed (section 2.5.2). Advantages of 4-d food diaries are that portion sizes are often well described giving a more accurate estimation of the quantity of foods consumed, and detailed descriptions of the different foods can be given enabling more accurate coding. However, the small number of days of recording can mean that nutrients such as vitamin D may not be captured. Furthermore, food diaries are time consuming for the participant to complete and the investigator to analyse compared with FFQs. Both estimated food diaries and FFQs are subject to under-reporting which can introduce bias. In FFQs, respondents may report eating less healthy foods less frequently than they actually do, and in food diaries an individual may decide to alter their diet to make it simpler to record, or to cover up poor eating habits. Some authors have used a pre-defined cut-off for the ratio of reported energy intake to calculated Basal Metabolic Rate (BMR) to evaluate the overall bias to underreporting (Goldberg *et al*.

1991). This technique is often used to correct for under-reporting energy intake but is not appropriate for evaluating FFQs where only a few foods supply most of the intake as with calcium and vitamin D. Adjustment for under-reporting of energy would inflate values, and excluding participants on the basis of under-reporting energy intake would introduce selection bias. Consequently, no adjustments for reported energy intake were made, but in line with the EPIC study, it was decided to exclude FFQs that had fewer than 70% of the food items checked (Davey *et al*. 2003).

Table 2.8 Summary of potential dietary assessment methods to use in the D_2/D_3 and DRISK studies

Dietary Assessment Method	Strengths	Limitations
24-Hour Recall	• Relatively simple to analyse • Does not require literacy	• Will not provide a valid estimate of nutrient intake for nutrients which are not consumed every day. • Requires the presence of an interviewer
Food Frequency Questionnaire	• Quick, easy to administer and affordable • Can be used to assess diet over an extended period	• Does not provide a valid estimate of absolute intake in individuals, but is good for ranking individuals in terms of their nutrient intake in a population • Relies on accurate memory of the participant
Food diary	• Does not rely on memory • Several days of food intake can be recorded to provide valid estimates of nutrient intake for individuals	• Time consuming and costly to code foods and analyse data • High participant burden as time consuming • The burden of recording all foods consumed may influence what is consumed i.e. simpler meals may be cooked.
Diet History	• Good for obtaining reasonably accurate estimates of nutrient intake for individuals	• Very high investigator and participant burden • Time consuming and costly to code foods and analyse data

2.5.1 Food frequency questionnaire

A lifestyle questionnaire (**Appendix 9**) similar to that used in the European Prospective Investigation in Cancer (EPIC) study (Bingham *et al.* 2001), that has been validated for vitamin D intake in the UK population (Crowe *et al.* 2011), was chosen for the D_2/D_3 study and DRISK study. The FFQ part of the questionnaire asked about the average frequency of consumption of 130 food and beverage items in the preceding 12 months with 9 frequency options ranging from never or less than once per month to 6+ times per day. The same questionnaire was also used in the CRESSIDA (section 2.6.1) and MARINA (section 2.6.2) studies.

2.5.2 4-d food dairy

A 4-d food dairy was used in the CRESSIDA study (section 2.6.1) to assess dietary intake at baseline and 12 wk. Foods entered were coded by a study investigator and the vitamin D intake estimated using WISP version 3.0.

2.6 Outline of previous studies on which further analysis of vitamin D status and vascular function measures were made

2.6.1 CRESSIDA

2.6.1.1 Objective

The CRESSIDA (**C**ardiovascular risk **RE**duction **S**tudy: **S**upported by an **I**ntegrated **D**ietary **A**pproach) compared effects on CVD risk factors and factors associated with the development of CVD of the UK dietary guidelines (DG) and a control based on a conventional British dietary pattern. Using a parallel-designed randomised controlled trial in 165 healthy non-smoking men and women aged 40-70 y, measurements were made of ABP on five occasions, and vascular function and CVD risk factors at baseline and after 12 wk, following random assignment to treatment. The primary outcomes were changes in 24 h day-time SBP and TC:HDL-C and secondary outcomes were changes in FMD, carotid to femoral PWV, hsCRP and a measure of insulin sensitivity (RQUICKI). The DG diet had a reduced salt and saturated fatty acid content, an increased proportion of wholegrain cereals, at least 5 portions of fruit and vegetables/d and advice to consume 1-2 portions of oily fish/wk. Measurements of dietary intake were made by 4-d food diaries (section 4.5.2) and by FFQ (section 4.5.1).

Blood samples were collected at the beginning and end of the intervention and were available for vitamin D analysis.

2.6.1.2 Dietary intervention

The main dietary goals for the control and cardioprotective diet are displayed in **Table 2.9**. Both diets were designed to provide 35% energy as fat. The DG diet aimed to supply approximately 10% saturated fatty acids (SFA); 16% monounsaturated fatty acids (MUFA), 6% polyunsaturated fatty acids (PUFA) and <1 % trans fatty acids (TFA). Salt intake was restricted to <6 g/d, and participants were asked to consume 5 portions of fruit and vegetables/d, two portions of fish/wk (one of which should be oily) and wholegrain cereal accounting for >50 % cereal intake. They were also given restrictions on intake of added sugars. The control diet aimed to supply approximately 14% SFA, 12% MUFA, 6% PUFA and <1% TFA, 3 portions of fruit and vegetables/d, oily fish less than 1 serving/month and no restriction on salt intake or added sugars. No advice was given to restrict the intake of food energy.

Table 2.9 Summary of the main dietary goals of the control and UK dietary guidelines diets

Diet constituent	Control	UK dietary guidelines diet (DG)
Salt	Not restricted	< 6 g/d
Saturated fatty acids	~ 13% energy	< 10% energy
Fruit and vegetables	3 serves / d	5 serves/d
Oily fish	<1 serve/ mo	1-2/ wk
Wholegrain	Refined cereals	> 50% cereal intake
Added sugars	Not restricted	Restricted
Total fat	~ 35% energy	~ 35% energy

Each group was provided with some foods in order to help them comply with the dietary advice they received. For example, to encourage subjects following the DG diet to increase their oily fish consumption, they were reimbursed for fresh oily fish that they purchased and were provided with tinned oily fish. Subjects following the control

diet were provided with tins of tuna and discouraged from consuming oily fish. Reinforcement of dietary advice was given throughout the study by a dietician.

2.6.1.3 Study design

The study was approved by the St Thomas' Hospital NHS Research Ethics Committee (Ref: 10/H0802/24), was formally registered as a randomised controlled trial (ISRCTN 92382106 http://www.controlled-trials.com/ISRCTN92382106), and received NHS R&D approval from the Guy's and St Thomas' Hospitals Foundation Trust. The study PIs were Professor Tom Sanders, Professor Philip Chowienczyk and Dr Wendy Hall.

A two-arm parallel design was used (**Figure 2.14**) and participants were randomly allocated to the control or DG diet for 12 wk by a computer programme using minimisation with age, gender, ethnicity and BMI. Recruitment started in 2010 with the first participant randomised in August 2010, and the last participant completing the study in July 2012.

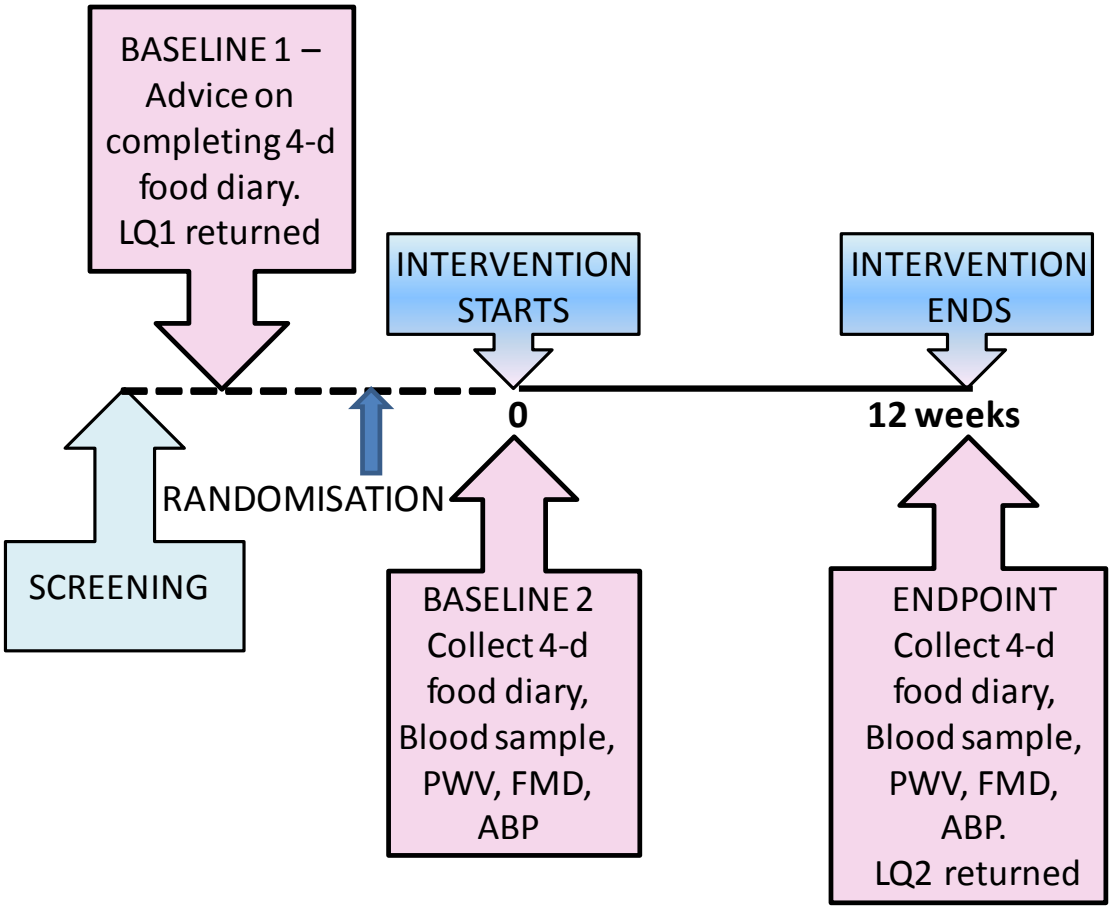


Figure 2.14 CRESSIDA study design. LQ, lifestyle and food frequency questionnaire; PWV, pulse wave velocity; FMD, flow mediated dilatation; ABP, 24-h ambulatory blood pressure monitoring.

2.6.1.4 Participants

Non-smoking men and women aged 40-70 y were recruited through newspaper and magazine advertisements, recruitment databases and an email circular to KCL staff and students. A fasting blood sample was taken at the screening appointment to determine that the subject's serum lipids (TC:HDL-C ratio <6.0, triglycerides <5.0 mmol/L, TC <8.0 mmol/L), liver function, glucose (<7.0 mmol/L) and haematology were within prescribed limits, and a urine sample was tested for cotinine, to exclude subjects with unreported smoking habit. Exclusion criteria included a reported history of angina, myocardial infarction or stroke, clinical history of cancer (excluding basal cell carcinoma) in the past 5 y, uncontrolled type 2 diabetes mellitus (fasting plasma glucose >7mmol/L), type 1 diabetes mellitus, chronic renal, liver or inflammatory bowel disease, history of substance abuse or alcoholism, pregnancy, unwilling to

refrain from the use of dietary supplements, unwilling to restrict consumption of oily fish, medication for high BP or serum cholesterol, weight change of >3 kg in preceding 2 months, BMI <20 and >35 kg/m^2 and an overall risk of CVD over the next 10 y of >20% (www.qrisk.org) in combination with untreated high BP or raised cholesterol.

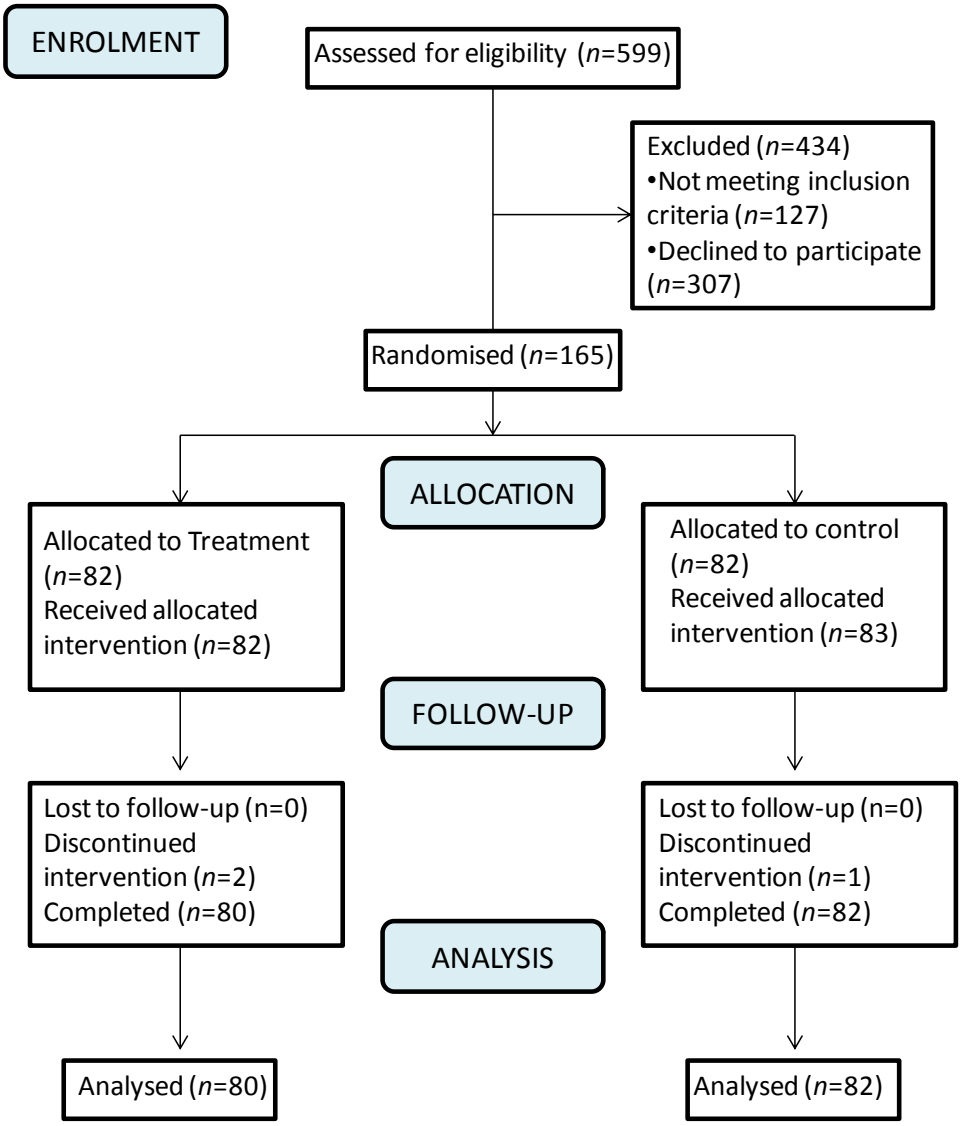


Figure 2.15 CRESSIDA CONSORT follow diagram

In total, 599 subjects were assessed for eligibility (**Figure 2.15**), and of these, 165 eligible subjects agreed to be randomised to the control or DG diet. Drop-out rates were very low with only 3 subjects not able to complete the study, leaving complete data on 162 subjects for analysis.

2.6.1.5 Outcome measurements

The first baseline visit took place at the metabolic unit in Franklin-Wilkins Building, KCL. ABP procedures were fully explained, including how to fit, start and stop the monitor, and the participant was given a monitor, that was later returned in person or by courier, which they were asked to wear for the next 25 hours. A 4-d food dairy and instructions for completing it were given by a registered Dietician, and the participant was asked to complete it before the next appointment. The second baseline and 12 wk visits at which vascular measurements were made took place at the CRF at St Thomas' Hospital. Participants wore another ABP monitor for 25 hours at least 2 days before these appointments (section 2.2.4). They were requested to avoid alcohol and vigorous exercise the day before, and to consume a meal low in fat and salt, provided by the study, the night before. They were asked to consume nothing but water after 2200 the night before and to arrive at the visits fasted. Measurements of weight, height and body composition were made, followed by the collection of a venous blood sample. After resting in the supine position for 15 min, supine BP was recorded using an automated sphygmomanometer (Omron 70CP or equivalent auto arm BP monitor). Up to six measurements were made ensuring that the coefficient of variation was less than 5% for 3 measurements in order for the results to be acceptable. Following this, the participant walked a short distance to an ultrasound room where they rested in the supine position for 30 min, before a trained vascular sonographer measured FMD of the brachial artery (the method was the same as that described in section 2.2.1). Following the vascular assessments, subjects were given breakfast followed by structured advice by a registered Dietician to follow the diet they had been randomly allocated to. They were provided with some of the foods and recipes to help with compliance, and a 4-d food diary which had been completed prior to the visit was checked. TC, HDL-C, LDL-C, triglycerides and hsCRP were amongst the analytes measured from the blood samples. Participants were requested to stop taking any nutritional supplements for the duration of the study.

2.6.2 MARINA

2.6.2.1 Objective

The MARINA (**M**odulation of **A**therosclerosis **R**isk by **I**ncreasing dose of **N**-3 fatty **A**cids) study aimed to test the effect of intakes of n-3 long chain polyunsaturated fatty acids,

(LC-PUFAs) equivalent to the consumption of 1, 2 or 4 portions of oily fish/wk, on endothelial function and arterial stiffness (Sanders *et al.* 2011). The primary outcome was endothelial function measured by FMD and the secondary outcomes were changes in arterial stiffness measured as carotid-femoral PWV, and 24 h ABP. Measurements of dietary intake were made by FFQ. Blood samples collected at baseline were available for vitamin D analysis.

2.6.2.2 Study design

Ethics approval was obtained from the St Thomas' Hospital NHS Research Ethics Committee (Ref: 08/H0802/3). The study was formally registered as a randomised controlled trial (ISRCTN66664610 http://www.controlled-trials.com/ISRCTN66664610), and received NHS R&D approval from the Guy's and St Thomas' Hospitals Foundation Trust. Full study details have been published (Sanders *et al.* 2011), but this thesis used only the baseline data from the study, so only the methods relevant to this will be included here. The study investigators were Professor Tom Sanders, Dr. Wendy Hall, Dr. Fiona Lewis, Dr. Philip Chowienczyk and Dr. Zoitsa Maniou. A parallel design was used (**Figure 2.16**) and participants were randomly allocated to 1 of 4 intervention groups by a computer program using minimisation with age, gender and ethnicity. The intervention was blends of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) providing 1.8, 0.9 or 0.45 g EPA + DHA, or placebo containing refined olive oil. Each treatment included a run-in period whereby participants took 3 placebo capsules/d for 1 month and a 12 month intervention phase (3 capsules/d). In this period, participants were also asked to not eat oily fish more than once per month. Recruitment commenced in May 2008, and the study was completed in September 2010.

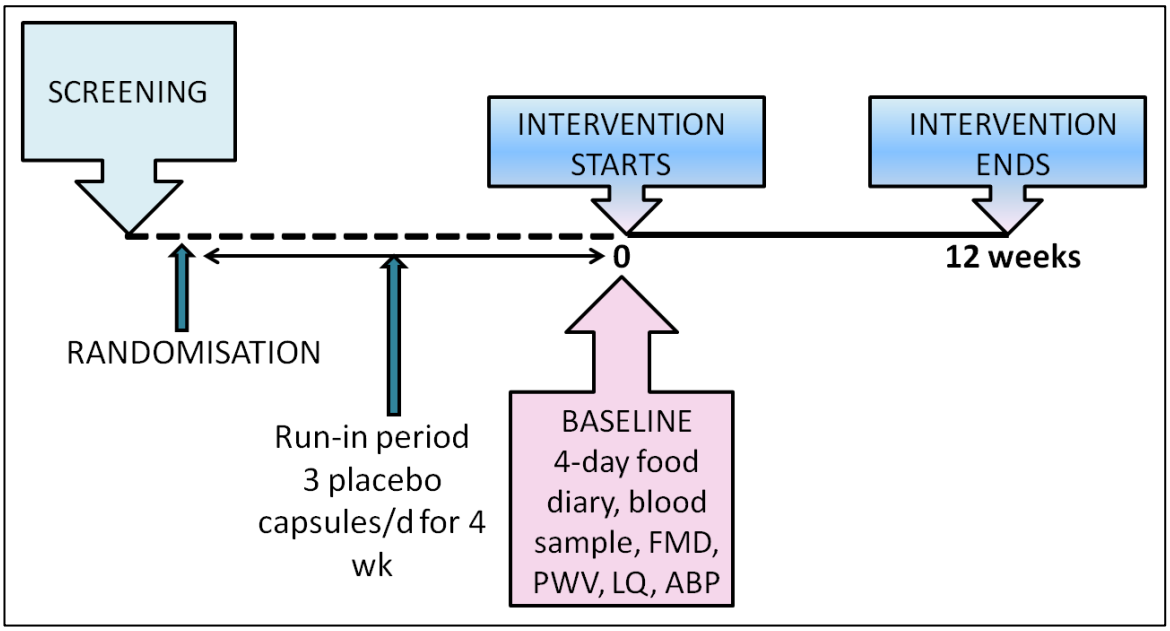


Figure 2.16 MARINA study design. LQ, lifestyle and food frequency questionnaire; PWV, pulse wave velocity; FMD, flow mediated dilatation; ABP, 24 h ambulatory blood pressure monitoring.

2.6.2.3 Participants

Non-smoking men and women aged 45-70 y were recruited from advertisements in newspapers, the study website, and an email circular to KCL staff and students. A fasting blood sample was taken at the screening appointment to determine that their serum lipids (TC:HDL-C ratio <6.0, triglycerides <5.0 mmol/L, TC <8.0 mmol/L), liver function, glucose (<7.0 mmol/L) and haematology were within prescribed limits. Exclusion criteria included a medical history of CVD, an overall risk of cardiovascular disease over the next 10 y of >20% assessed according to QRISK2 (www.qrisk.org), cancer (excluding basal cell carcinoma) in the previous 5 y, type 1 diabetes mellitus, uncontrolled type 2 diabetes (fasting plasma glucose >7mmol/L), chronic renal, liver, or inflammatory bowel disease, history of substance abuse of alcoholism, pregnancy, weight change of >3kg in preceding 2 months, and BMI <20 and >35 kg/m^2. After 475 screening appointments, 367 subjects were randomly assigned to treatment. In the run-in period, a total of 25 participants withdrew from the study, leaving data on 342 participants for the cross-sectional analyses conducted in this thesis.

2.6.2.4 Outcome measurements

Baseline measurements were made after the run-in period and participants were asked to avoid strenuous exercise, caffeine, alcohol from 1400 on the previous day up until the time of the visit. Participants were also requested to avoid foods high in fat and to consume a low-fat frozen meal (<10g fat) before 2200 the day before. They were asked to consume nothing but water after 2200 and to arrive at the visit fasted. A FFQ and lifestyle questionnaire (**Appendix 9**) was sent to the participant with the run-in capsules, completed to assess nutrient intake over the previous 12 months, before the run-in period, and returned at the visit. Measurements of weight, height and waist circumference were made at the beginning of the visit and a venous blood sample collected. After a rest in the supine position for 30 min, a trained vascular sonographer measured FMD of the brachial artery (the method was the same as that described in section 2.2.1). Subsequently, the participant rested supine for 15 min before having their arterial stiffness measured using the SphygmoCor VW apparatus with SphygmoCor analysis software (SphygmoCor version 7.01 AtCor Medical Pty) (section 2.2.2.2) and then Vicorder (Skidmore Medical, Bristol, UK) (section 2.2.2.1). At the end of the visit, participants were fitted with an ABP monitor (A & D TM-2430 devices from A & D Instruments) that they were asked to wear for the next 25 hours (section 2.2.4). TC, HDL-C, LDL-C and triglycerides were measured from the blood sample. Subjects were requested to stop taking any nutritional supplements for the duration of the study.

Chapter 3

Influences on serum 25-OH-D concentrations and vascular outcomes

3.1 Introduction

Cross-sectional and prospective cohort studies have found a higher prevalence of CVD in people with lower serum concentrations of 25-OH-D (Kendrick *et al.* 2009; Kim *et al.* 2008). It is unknown what mechanisms are responsible for these associations, but vitamin D may have effects on CVD risk markers such as arterial stiffness, endothelial function, BP, inflammatory markers and lipids. Whilst many cross-sectional studies measuring serum 25-OH-D and these indices have been published in recent years, they have shown inconsistent results (Al Mheid *et al.* 2011; Pirro *et al.* 2012; Chacko *et al.* 2011; Gepner *et al.* 2014; Jablonski *et al.* 2011; Rezai *et al.* 2011), and only a limited number have measured a range of indices of vascular function and markers of inflammation in a large cohort of healthy participants. Furthermore, the majority of studies that have measured BP have used a clinic reading, rather than an ambulatory 24 h measurement which is more accurate (Coats *et al.* 1992).

Serum 25-OH-D concentrations are strongly influenced by UVB exposure in addition to dietary intake. The ability to synthesise 25-OH-D from sunlight is dependent on exposure (latitude, time of day, season, duration of exposure), the area of skin exposed and skin pigmentation. Vieth *et al.* (Vieth *et al.* 2007b) argue that recommended dietary intakes are unlikely to maintain optimal serum 25-OH-D concentrations and that either UVB exposure, which has the adverse effect of increasing risk of skin cancer, or high dose supplementation are required. However, there is limited published data on the effect of dietary intakes in line with recommendations on serum 25-OH-D concentrations. Whilst there are currently no UK recommendations for adults, 10 µg is the amount recommended for those confined indoors with little exposure to sunlight (Scientific Advisory Committee on Nutrition 2007). There is no differentiation between men and women despite differences in body weight. Oily fish is the richest source of vitamin D in the diet providing 5-16 µg/100 g, and the Scientific Advisory Committee on Nutrition (SACN) has previously advised that people 'eat at least two portions of fish, of which one should be oily, weekly' (Scientific Advisory Committee on Nutrition 2004). Despite this, the latest National Diet and Nutrition Survey (NDNS) figures from between 2008 and 2010 show that the mean consumption of oily fish in the UK was 58 g/wk, which is much lower than the recommendation of at least one portion (140 g)/wk in 19-64 y olds, largely

due to only 25% of the population being consumers (Beverley Bates 2011). In the UK, margarine and some breakfast cereals, cheeses, yoghurts are fortified with vitamin D at around only 0.5-2.5 μg/average portion, but unlike the US, the UK does not fortify milk (Expert Group on Vitamins and Minerals 2003a).

An opportunity to assess cross-sectional relationships between serum 25-OH-D and indices of vascular function and inflammation was provided by data from the CRESSISA and MARINA trials where measurements of vascular function, 24 h ABP and hsCRP had been taken. The trials recruited healthy non-smoking participants aged 40-70 y who had not had a CVD event. The former study also allowed an assessment of the impact on vitamin D status of advice to increase oily fish consumption.

3.2 Methods

3.2.1 Hypothesis

Primary hypothesis

Low vitamin D status measured as serum 25-OH-D concentrations is associated with higher 24 h BP, increased arterial stiffness measured as PWV and impaired endothelial function measured as FMD.

Corollary

The variability in serum 25-OH-D concentrations is determined by usual dietary vitamin D intake as well as UVB exposure and skin pigmentation.

3.2.2 Objective

To measure 25-OH-D concentrations in the 2 RCTs and estimate the influence of usual vitamin D intake on these. To investigate associations between 25-OH-D and factors including age, BMI, physical activity, gender and skin pigmentation, and to look for associations between vitamin D status and 24 h BP, PWV and FMD adjusting for potential confounders.

3.2.3 Study design

Details of the study design, ethics approval, primary and secondary outcomes, inclusion and exclusion criteria, visits and outcome measurements can be found in

Chapter 2 for the two trials; section 2.6.1 for CRESSIDA and section 2.6.2 for MARINA. The baseline data from both studies have been used in this chapter to investigate the relationship between 25-OH-D concentrations and CVD risk markers. In both studies, endothelial function was measured using the FMD technique (section 2.2.1), arterial stiffness as PWV by SphygmoCor (ArtCor Medical, Sydney, Australia) (section 2.2.2.2) and BP by 24 h ABP monitoring. Anthropometric measurements of weight, height and waist circumference were made and a venous blood sample taken to determine TC, HDL-C, LDL-C, triglycerides and hsCRP using identical methods to those described in Chapter 2. The CRESSIDA study recruited men and women aged 40-70 y and the MARINA study men and women aged 45-70 y. The percentage of women who were post-menopausal was 53% in the CRESSIDA study and 71% in the MARINA study. The CRESSIDA study took place August 2010-July 2012, and the MARINA study May 2008-July 2009. For the purposes of this chapter, the 25-OH-D content of the serum in both trials was determined using a chemiluminescent immunoassay (section 2.4.6.2) by KingsPath, a CPA laboratory of the Department of Clinical Biochemistry at KCH, London. Serum had been stored at -70˚C prior to analysis.

The CRESSIDA study compared effects on CVD risk factors of the UK dietary guidelines (DG) to a control based on a conventional British dietary pattern. The DG diet had a reduced salt and saturated fatty acid content, an increased proportion of wholegrain cereals, at least 5 portions of fruit and vegetables/d and advice to consume 1-2 portions of oily fish/wk. Additional data from this study at the follow-up (12 wk) appointment were used to investigate changes in vitamin D status due to increased oily fish consumption. A simplified study design for this RCT is displayed in **Figure 3.1.** Serum 25-OH-D concentrations from the 12 wk appointment were also determined.

Subjects in both studies were asked to not take any supplements, including multivitamins, cod liver oil and fish oil capsules for the duration of the study.

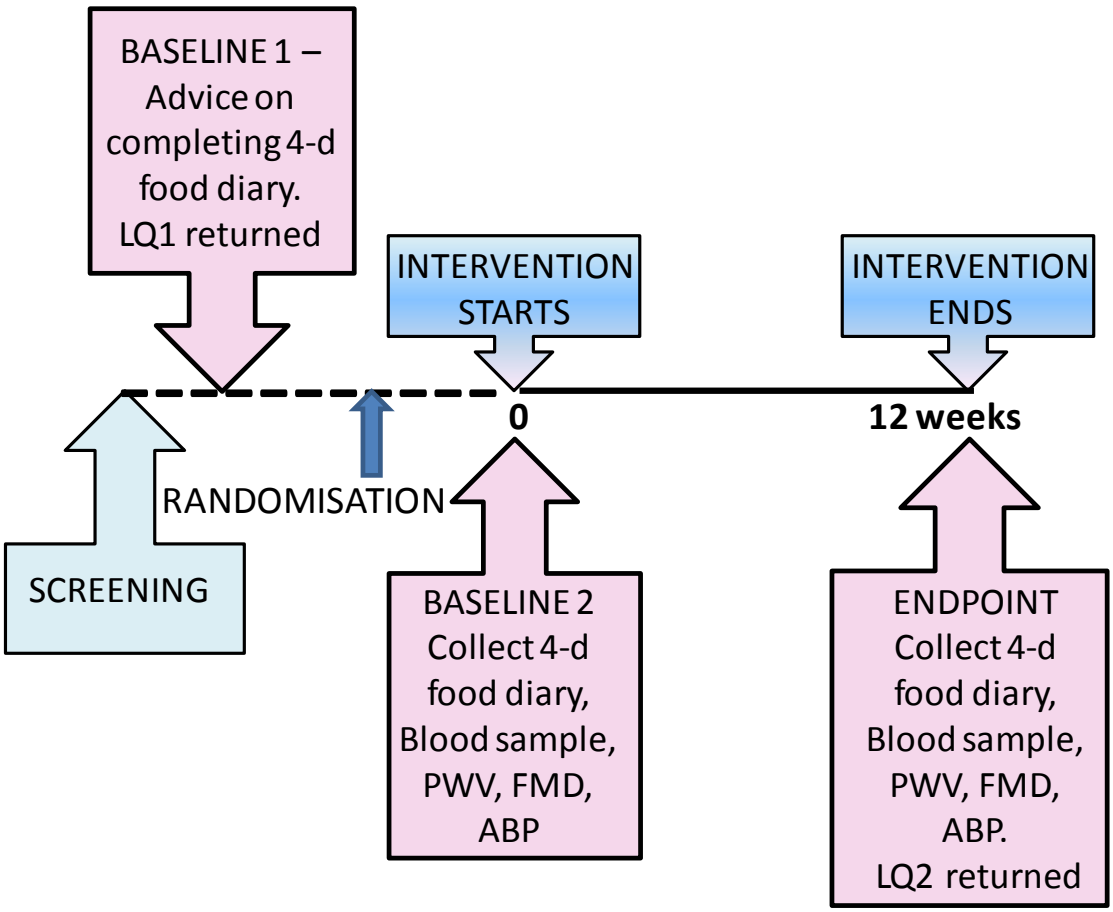


Figure 3.1 CRESSIDA study design. LQ, lifestyle and food frequency questionnaire; PWV, pulse wave velocity; FMD, flow mediated dilatation; ABP, 24 h ambulatory blood pressure monitoring.

3.2.4 Dietary intake

Diet was assessed at baseline in the MARINA study by a FFQ contained in a lifestyle questionnaire (LQ, section 2.5.1, **Appendix 9**) which assessed nutrient intake over the previous 12 months (before the 1 month run-in period when participants took 3 placebo capsules/d that did not contain vitamin D). The FFQ data were analysed as described in section 4.2.7 to determine nutrient intake per day. In the CRESSIDA study, diet was assessed by 4-d food diaries at baseline and 12 wk (section 2.5.2). These were coded and analysed using WISP version 3.0 by a CRESSIDA study investigator. The main specifics of the DG and control diet are summarised in section 2.6.1.2, but differences between the two diets in terms of vitamin D intake are covered here. Participants in the DG group were requested to consume two portions of fish/wk (one of which

should be oily), whereas participants in the control group were requested to consume less than 1 serving/month of oily fish. The DG group was reimbursed for fresh oily fish that they purchased and were provided with tinned oily fish (sardines, salmon or mackerel). The control group were provided with tins of tuna and discouraged from consuming fresh and other tinned oily fish. The guidance for the DG group was to consume no more than 3 eggs/wk, but the control group could consume unrestricted eggs. The DG group was given non-fortified cereals, and fortified margarine containing approximately 0.75 μg/100 g, whereas the control group received fortified cereals containing approximately 4 μg/100 g and a spread which was not fortified.

3.2.5 Physical activity

Physical activity levels were calculated for the CRESSIDA and MARINA studies from data collected in the LQ (**Appendix 9**) which asked questions on how many hours per wk the participant spent on housework, DIY, gardening, walking, cycling and other physical activities, how many hours this exercise was practiced vigorously, and how many stairs the participant climbed per day on average. Physical activity was calculated by multiplying the duration of each level of activity by previously determined physical activity ratios (Committee on Medical Aspects of Food Policy 1991), and was expressed as Metabolic Equivalents (MET)/d. A resting metabolic rate was assumed to have an MET of 1.

3.2.6 Statistical analysis

Where values were below the limit of detection, the limit of detection was assigned. Statistical analysis of the data was conducted using SPSS for Windows Version 21.0. Standard distributional checks were made, and where appropriate, analyses were attempted following log transformation. Where variables could not be normalised, medians and inter-quartile ranges were determined and non-parametric tests performed. In order to test whether the two studies differed at baseline, comparisons were made using the independent samples *t*-test, Mann-Whitney *U* test or Pearson's chi-squared test for categorical variables. Associations between factors were determined using partial correlations. Comparisons between groups were made using univariate ANOVA for normally distributed variables or the Mann-Whitney *U* test or Kruskal-Wallis test for variables that were not normally distributed.

3.3 Results

3.3.1 Subject characteristics

In the CRESSIDA study, 599 subjects were assessed for eligibility, and of these 165 eligible subjects were randomised to the control or DG diet (see section 2.6.1.4). A total of 3 subjects dropped out, leaving complete data on 162 subjects; *n*=82 in the control group and *n*=80 in the DG diet group. In the MARINA study, following 475 screening appointments, 367 subjects were randomly assigned to treatment (see section 2.6.2.3). In the one month run-in period, a total of 25 participants withdrew from the study, leaving data on 342 participants at baseline. Baseline characteristics for both studies are displayed in **Table 3.1**. The MARINA subjects were significantly older and had a correspondingly higher PWV than the CRESSIDA participants. Waist circumference was significantly greater in CRESSIDA participants, but there was no difference in BMI between the studies. There were no other significant differences between studies.

Table 3.1 Subject characteristics at baseline for the CRESSIDA (*n*=165) and MARINA (*n*=342) studies

	CRESSIDA (*n*=165)	MARINA (*n*=342)	Combined (*n*=507)
Age (y)*	52 (8)	55 (7)	54 (7)
Gender (M/F)	65/100	131/211	196/311
Ethnicity			
White	137 (83.0%)	281 (82.2%)	418 (82.4%)
Black	16 (9.7%)	18 (5.3%)	34 (6.7%)
Asian	8 (4.8%)	19 (5.6%)	27 (5.3%)
Far East	2 (1.2%)	9 (2.6%)	11 (2.2%)
Other	2 (1.2%)	15 (4.4%)	17 (3.4%)
Height (m)	1.66 (0.24)	1.69 (0.10)	1.68 (0.16)
Weight (kg)	75.0 (13.7)	73.7 (15.8)	74.1 (15.2)
BMI (kg/m^2)	26.1 (3.8)	25.6 (4.3)	25.7 (4.2)
Waist circumference (cm)*	92.7 (11.8)	88.8 (11.5)	90.1 (11.7)
Total cholesterol (mmol/L)	5.3 (1.0)	5.4 (1.1)	5.4 (1.1)
HDL-Cholesterol (mmol/L)†	1.5 (0.4)	1.6 (0.5)	1.5 (0.5)
LDL-Cholesterol (mmol/L)	3.2 (0.8)	3.2 (0.8)	3.2 (0.8)
Total:HDL cholesterol ratio	3.5 (0.9)	3.5 (1.0)	3.5 (1.0)
Triglycerides (mmol/L)†	1.2 (0.6)	1.2 (0.7)	1.2 (0.6)
HsCRP mg/L‡	1.00 (0.30,2.00)	0.70 (0.30,2.10)	0.80 (0.30,2.00)
Physical activity (MET/d)‡	39.7 (37.4,43.5)	41.4 (38.6,45.1)	40.7 (37.9,44.2)
Pulse Wave Velocity (m/sec)†*	7.4 (1.2)	8.8 (1.8)	8.3 (1.7)
Flow mediated dilation (%)	5.5 (3.1)	5.4 (3.0)	5.4 (3.0)
24-h SBP (mm Hg)	122 (12)	124 (13)	123 (13)
24-h DBP (mm Hg)	74 (7)	75 (7)	74 (7)

Results as n(%), mean (SD), †geometric mean (approx SD) or ‡median(IQR). HsCRP, high sensitivity C-reactive protein; SBP, systolic blood pressure; DBP, diastolic blood pressure. *Significant difference between CRESSIDA and MARINA studies (P<0.001). No other significant differences between studies tested using the independent samples *t*-test for normally-distributed variables, Mann-Whitney *U* test for not-normally distributed continuous variables, and Pearson's chi-squared test for categorical variables were found. Data were missing for some outcomes; for CRESSIDA, MARINA and both studies combined, the numbers of participants included were 153, 342 and 495 for FMD, 163, 342 and 498 for PWV, 163, 327 and 490 for BP and 162, 342 and 504 for hsCRP, respectively.

3.3.2 Vitamin D intake and status

At baseline, mean (SD) concentrations of serum 25-OH-D were 39.3 (24.1) nmol/L in men and 35.2 (21.2) nmol/L in women for both studies combined. The percentage of participants who had serum 25-OH-D concentrations ≤25 nmol/L and ≤50 nmol/L were 21.4% and 67.6%, respectively (**Figure 3.2).** Spearman's rank correlation coefficients between vitamin D intake (μg/d) and serum 25-OH-D concentrations (nmol/L) were strongest (ρ = 0.277, P=1.6x10^{-7}) in the CRESSIDA study in which vitamin D intake was assessed by 4-d food diaries, but still highly significant in the MARINA study in which a FFQ was used to assess intake (ρ = 0.160, P=0.003). Mean vitamin D intakes for increasing serum 25-OH-D categories are displayed in **Table 3.2** for both studies. A significantly greater mean intake was found in the highest (75+ nmol/L) compared to the lowest (25-50 nmol/L) 25-OH-D category in the CRESSIDA study and in the 50-75 nmol/L category compared to the 0-25 nmol/L category in the MARINA study. The non-significant decrease in the mean intake from 3.9 μg/d in the 50-75 nmol/L category to 3.3 μg/d in the 75+ nmol/L group in the MARINA study could be a play of chance as there were only 10 participants in that category. The other possible difference is that a FFQ was used *vs*. 4-d food diaries in CRESSIDA. However, estimates of vitamin D intake from FFQs and 4-d diaries did not differ significantly in the CRESSIDA study (D. Reidlinger and TAB Sanders, personal communication).

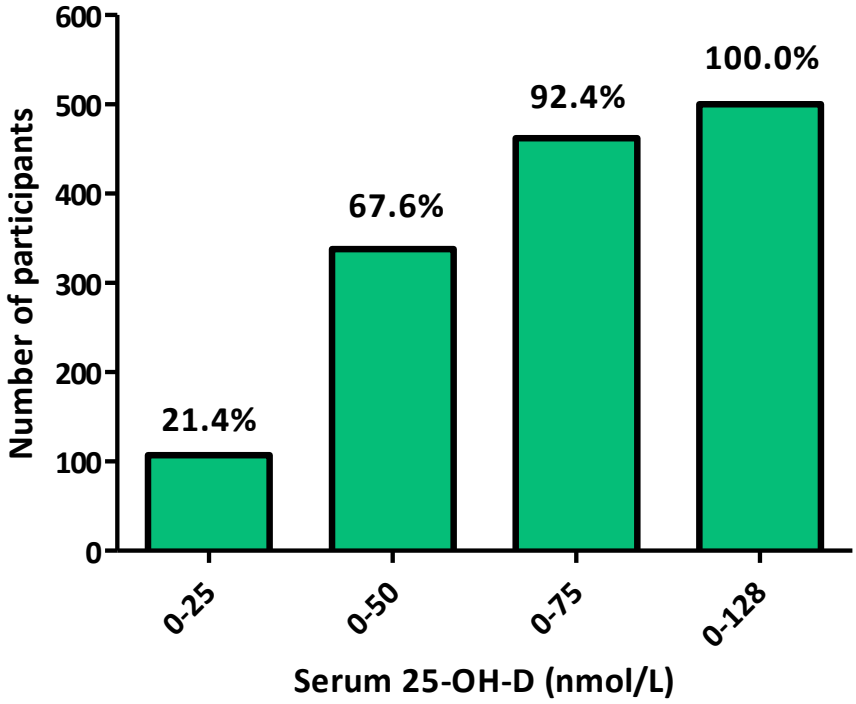


Figure 3.2 Cumulative number of participants in categories of serum 25-OH-D in the CRESSIDA and MARINA studies (cumulative percentages of participants shown above bars)

Table 3.2 Mean vitamin D intake (µg/d) according to vitamin D status (serum 25-OH-D) in the CRESSIDA and MARINA studies

	Vitamin D category (nmol/L)				*P* value†
	0-25	25-50	50-75	75+	
CRESSIDA	-	*n*=69	*n*=65	*n*=28	
		2.3 (1.7,2.8)	3.2 (2.7,3.8)	4.0 (3.1,4.9)	<0.001
MARINA	*n*=107	*n*=162	*n*=59	*n*=10	
	3.0 (2.6,3.4)	3.3 (2.9,3.6)	3.9 (3.4,4.4)	3.3 (2.0,4.5)	0.006

Values are mean (95% CI). Vitamin D intake was assessed using 4-d estimated food diaries in the CRESSIDA study and FFQs in the MARINA study. †Categories were compared using a Kruskal-Wallis test.

3.3.3 Effect of BMI, age, physical activity, gender and skin pigmentation on serum 25-OH-D concentrations

A significant difference was found in mean serum 25-OH-D concentrations between categories of BMI for both CRESSIDA and MARINA studies combined (*P*=0.001); people in the obese but not overweight category had a lower vitamin D status. There were no significant differences in age or physical activity between the categories 0-25, 25-50, 50-75 and 75+ nmol/L of serum 25-OH-D. However, there were significant differences in vitamin D status between males and females (*P*=0.019) and between people with white and melanised skin (*P*=2.06 x 10^{-7}) (**Table 3.3**), although these were not due to differences in vitamin D intake.

Table 3.3 Differences in serum 25-OH-D concentrations between BMI categories in participants aged 40-70 y

BMI (kg/m^2)	25-OH-D (nmol/L)†
18.5-25.0 *n*=238	45 (43, 48)
25.0-30.0 *n*=187	43 (40, 47)
30.0-35.0 *n*=82	35 (30, 40)
P value	0.001

†Marginal means with 95% confidence intervals adjusted for age and gender. *P* value from univariate ANOVA.

Table 3.4 Differences in serum 25-OH-D concentrations between men and women and people with white and melanised skin in participants aged 40-70 y

	Vitamin D intake (µg/d)‡	25-OH-D (nmol/L)†
Gender		
Female *n*=306	2.6 (1.6-4.0)	41 (39, 44)
Male *n*=194	2.8 (1.8-3.9)	46 (43, 49)
P value	0.374	0.019
Skin pigmentation		
White *n*= 429	2.4 (1.1-4.1)	45 (43, 47)
Melanised skin *n*= 71	2.7 (1.1-4.1)	30 (25, 35)
P value	0.151	2.06 x 10^{-7}

†Marginal means with 95% confidence intervals adjusted for age and BMI, and also by gender for skin pigmentation. ‡Median (IQR). *P* value from univariate ANOVA for 25-OH-D and Mann-Whitney *U* test for vitamin D intake.

3.3.4 Seasonal variability in 25-OH-D concentrations

The CRESSIDA and MARINA studies recruited participants throughout the year in different seasons. **Figure 3.3** shows that the mean concentrations of serum 25-OH-D increased from May to August for both studies combined with the highest concentrations occurring in August (53 nmol/L). There was a dip in vitamin D status in the winter months, with the lowest concentrations occurring in December (28 nmol/L). Univariate ANOVA with age, gender and BMI as covariates showed a significant difference in vitamin D status between quarters of the year (P=6.3 x 10^{-6}). Mean 25-OH-D concentrations were significantly higher July-September compared with every other quarter.

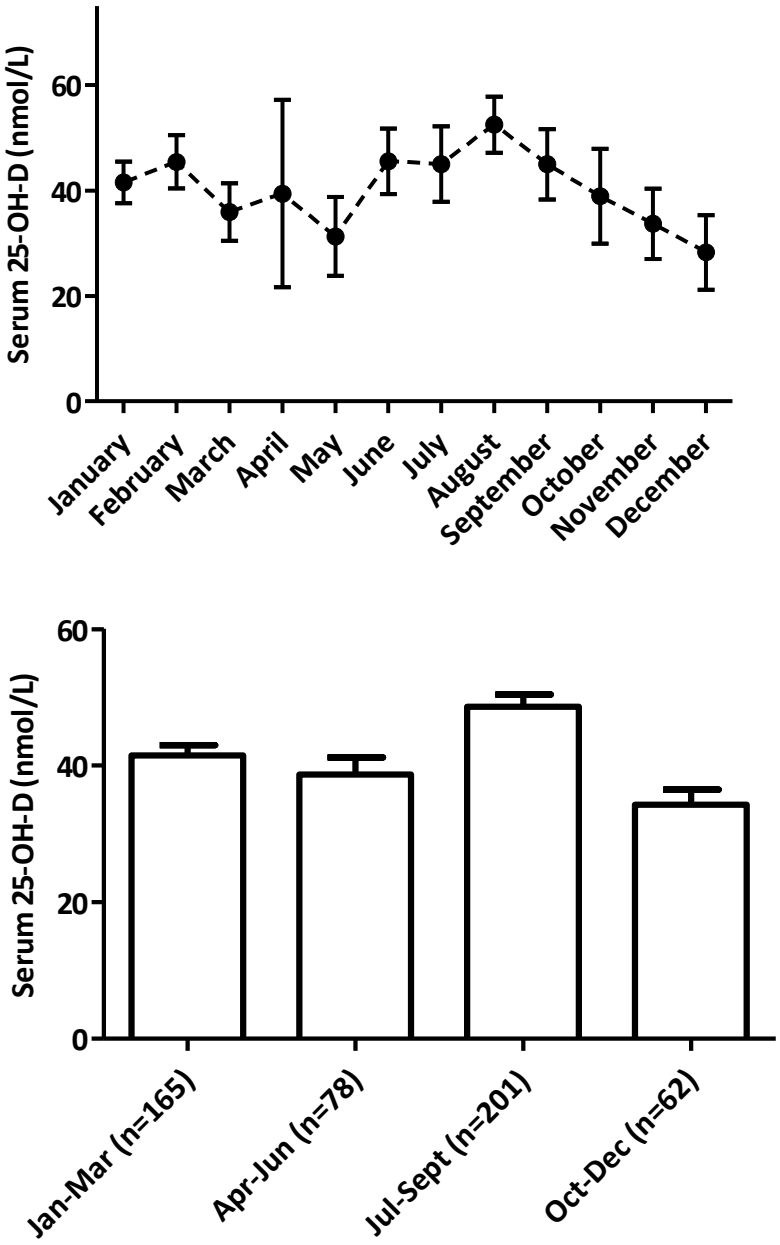


Figure 3.3 Mean (95% CI) serum 25-OH-D concentrations measured throughout the year at baseline in the CRESSIDA and MARINA studies combined.

3.3.5 Association between serum 25-OH-D and indices of vascular function and inflammation

Table 3.5 shows correlations between serum 25-OH-D, BP, endothelial function, arterial stiffness and hsCRP for the combined data from the CRESSIDA and MARINA studies. Inverse correlations with serum 25-OH-D concentrations were found for 24 h DBP (ρ =-0.10, P=0.03), hsCRP (ρ = -0.11, P=0.01) and PWV (ρ = -0.12, P=0.01).

Table 3.6 presents the results with the participants categorised according to their vitamin status adjusted for age, gender, BMI, ethnicity, physical activity and seasonality. There was a highly significant difference between serum 25-OH-D categories for PWV (P=4.57 x 10^{-6}) with, for example, participants in the 0-25 and 25-50 nmol/L categories having stiffer arteries compared with those in the 75+ nmol/L category. There was also a significant linear trend for PWV across categories (P=1.41 x 10^{-6}). A significantly greater FMD was found in participants in the 75+ nmol/L category compared with the 50-75 nmol/L category suggesting that concentrations above 75+ may have beneficial effects. However, FMD decreased between the 0-25 and 50-75 nmol/L categories and the number of participants (n=36) in the 75+ group was small; therefore this may be a chance finding.

Table 3.5 Partial correlations between serum 25-OH-D concentrations and 24-h systolic (SBP) and diastolic (DBP) blood pressure, carotid to femoral pulse wave velocity (PWV), flow mediated dilation of the brachial artery (FMD) and high-sensitivity C-reactive protein (hsCRP) in 466 healthy men and women aged 40-70 y.

	r	*P*
24 h SBP (mm Hg)	-0.04	0.37
24 h DBP (mm Hg)	-0.10	0.03
HsCRP (mg/L)	-0.11	0.01
FMD (%)	-0.04	0.33
PWV (m/sec)	-0.12	0.01

Partial correlation coefficient adjusted for age, gender and study.

Table 3.6 24 h systolic (SBP) and diastolic (DBP) blood pressure, carotid to femoral pulse wave velocity (PWV), flow mediated dilation of the brachial artery (FMD) and C-reactive protein measured using a high sensitivity assay (hsCRP) in healthy men and women aged 40-70 y by category of vitamin D status.

	Serum 25-OH-D category (nmol/L)				*P* value
	0-25	**25-50**	**50-75**	**75+**	
	n=101	*n*=212	*n*=115	*n*=36	
24 h SBP (mm Hg)	124.0 (121.7,126.3)	122.8 (121.2,124.3)	123.8 (121.7,126.0)	120.9 (117.1,124.7)	0.47
24 h DBP (mm Hg)	75.5 (74.2,76.8)	73.9 (73.0,74.7)	74.6 (73.4,75.8)	72.4 (70.2,74.5)	0.06
HsCRP (mg/L)‡	2.3 (1.7,3.0)	2.0 (1.6,2.4)	1.5 (0.9,2.1)	1.1 (0.0,2.2)	0.178
PWV (m/sec)	9.0 (8.7,9.3)	8.6 (8.4,8.8)	8.1 (7.9,8.4)	7.7 (7.2,8.2)	4.6×10^{-6}
FMD (%)	5.7 (5.2,6.3)	5.3 (4.9,5.6)	4.8 (4.3,5.3)	6.5 (5.6,7.4)	0.005

Values are mean (95% CI); significance determined by multivariate ANOVA comparison between categories adjusted for age, gender, BMI, ethnicity, physical activity and seasonality. Significant differences exist between categories where confidence intervals do not overlap. ‡Median (IQR) and significance calculated using the Kruskal-Wallis test. There is a statistically significant linear trend for PWV (P=1.41 x 10^{-6}).

3.3.6 Effect of UK Dietary Guidelines (DG) diet on serum 25-OH-D concentrations

There were no differences between participants allocated to control and dietary guidelines (DG) groups at baseline and the mean 25-OH-D concentrations were 60.3 and 58 nmol/L respectively. Following the dietary intervention serum 25-OH vitamin D increased by 9.2 nmol/L (95% CI 4.2, 14.2; *P*<0.001) in DG group compared to the control group when adjusted for baseline 25-OH-D, age, BMI, gender, ethnicity and seasonality (**Figure 3.4**). The proportion of participants with serum 25-OH-D concentrations <50 nmol/L decreased by 11% in the DG group *vs* a 4% increase in the control group. Mean vitamin D intake increased from 3.0 μg/d to 6.6 μg/d in the DG group and decreased from 2.9 μg/d to 2.7 μg/d in the control group. Compared with the control group, vitamin D intake increased by 3.8 μg/d following the DG diet (95% CI 2.7, 5.0, *P*<0.001). Fish consumption was the main contributor to vitamin D intake in both groups at baseline (52% of total intake), followed by meat and meat products (10%), fats and oils (10%), cereals (10%) and eggs (7%) (**Figure 3.5**). The contribution of fish to vitamin D intake increased to 61% in the DG group at 12 wk. Fats and oils (predominantly the fortified study spread) were the next most important contributor (20% of total intake) in this group.

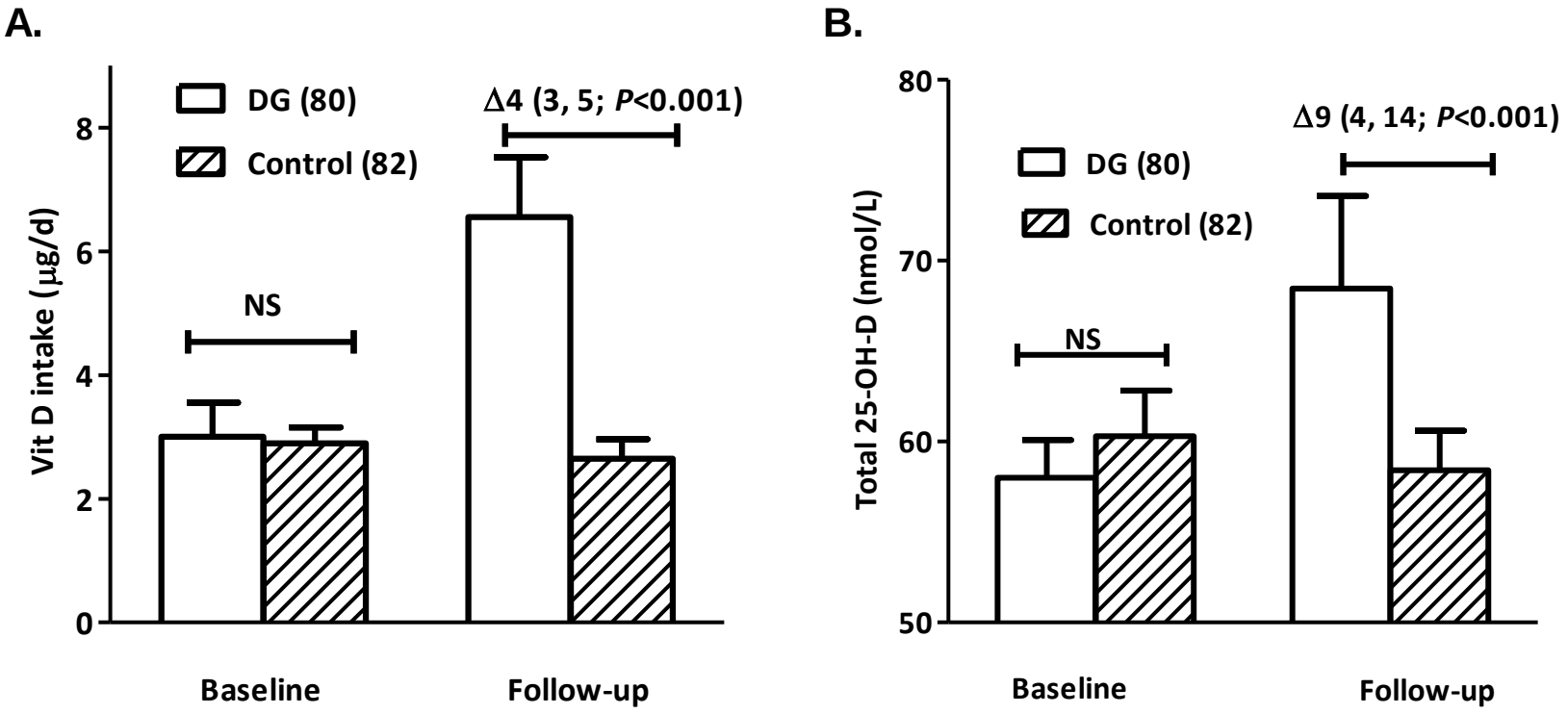


Figure 3.4 Changes in vitamin D intake and serum 25-OH-D concentrations from baseline to follow-up in the group which followed UK dietary guidelines (DG) compared to a control based on a conventional British dietary pattern. Bars are means with SEM. Changes are from univariate ANOVA on the 12 wk data, adjusted for baseline. The change for B. is also adjusted for age, BMI, gender, ethnicity and seasonality.

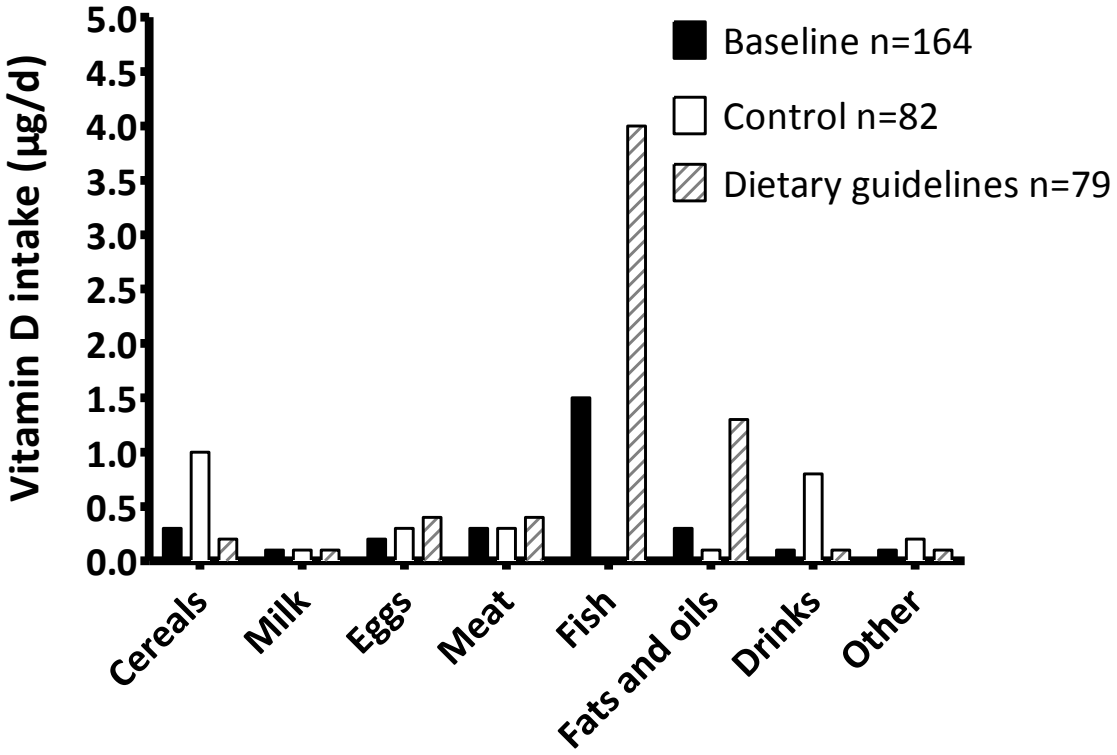


Figure 3.5 Comparison of the percentage contribution of different food groups to vitamin D intake at baseline in both groups, and at 12 wk in the group following UK dietary guidelines (DG) and the control based on a conventional British dietary pattern. Data is from 4-d food diaries.

3.4 Discussion and Conclusion

Of participants aged 40-70 y in the CRESSIDA and MARINA studies, 21.4% and 67.6% had serum 25-OH-D concentrations ≤25 nmol/L and ≤50 nmol/L respectively, and mean concentrations were 39.3 and 35.2 nmol/L for men and women respectively. The percentage of participants who were deficient (<25 nmol/L) is comparable to UK data from the most recent 2008-2012 National Diet and Nutrition Survey (NDNS) of 1769 individuals which found deficiency in 22.8% of adults aged 19-64 y and in 21.0% of adults aged 65+ y. However, mean concentrations were higher in the NDNS even though a lower percentage of blood samples (54% in adults aged 19-64 y and 47% in adults aged 65+ y) were taken in the summer months of April-September compared to in the CRESSIDA and MARINA studies (67%). They were 43.5 nmol/L and 47.3 nmol/L in men and women aged 19-64 y, and 47.0 nmol/L and 42.5 nmol/L in men and women aged 65+ y (Bates *et al.* 2014), respectively. This may be due to a lower percentage of people with melanised skin compared to 17% in the studies analysed here, but data on ethnicity has not yet been published for this NDNS survey for comparison. In the MARINA and CRESSIDA studies a significantly higher vitamin D status was found in whites compared with people with melanised skin, and in men compared to females. The finding for skin-type is consistent with the literature which shows a higher vitamin D status in whites compared with blacks and South Asians (Scragg *et al.* 1920; Kift *et al.* 2013). Dark skin may lead to a reduced capacity of the body to convert vitamin D to its active form as melanin skin pigmentation absorbs UVB photons and competes with 7-dehydrocholesterol for them (Holick *et al.* 1981). The higher serum 25-OH-D concentrations in men were not due to differences in vitamin D intake between men and women and so may be due to differences in UVB exposure, although physical activity levels did not differ between the genders, so if this is the case it would not be due to a greater level of outdoor sport/working in men. One study has found that an age-related decline in serum 25-OH-D concentrations occurred earlier in women than in men which could explain these findings as these were older adults aged 40-70 y (Maggio *et al.* 2005).

On examination of the seasonal variability of vitamin D status, the CRESSIDA and MARINA studies showed a marked seasonal change in serum 25-OH-D, with the highest concentrations measured in the summer months. This was to be expected due

to the increase in UVB exposure in the UK summer, and it has been documented previously in British cohorts (Hypponen and Power 2007; Zgaga *et al.* 2011). In addition to variability in vitamin D status due to differences in UVB exposure, dietary intake of vitamin D was found to be strongly correlated with vitamin D status (P=0.008) over a serum 25-OH-D range of 10 to 128 nmol/L, with participants in higher categories of 25-OH-D having larger dietary intakes of vitamin D. Furthermore, modification of the diet to conform to the UK dietary guidelines led to over a doubling of vitamin D intake from 3.0 µg/d to 6.6 µg/d, and an increase in serum 25-OH-D of 9.2 nmol/L (95% CI 4.2, 14.2, P<0.001) compared to the control group that was consuming a conventional British dietary pattern. No subjects were taking supplements and these findings were primarily in response to an increased consumption of oily fish after following the recommendations of 1-2 portions per wk compared to less than 1 serving/month in the control group. They also occurred despite the DG group being given non-fortified cereals whilst the control group received fortified cereals. The increase in 25-OH-D concentrations is about a third of that found in a study which gave participants orange juice fortified with 25 µg D_3/d for 11 wk (Biancuzzo *et al.* 2010). Although cross-sectional studies have shown oily fish to be a key contributor to vitamin D intake (Hill *et al.* 2004; Zgaga *et al.* 2011), to the author's knowledge there have been no other trials published of the effect of increased oily fish intake on vitamin D intake and status in the UK.

Cross-sectional analysis looking at the relationship between vitamin D status and CVD-related factors in the CRESSIDA and MARINA studies found a strong association between serum 25-OH-D concentrations and arterial stiffness; participants with a higher serum 25-OH-D concentration had a lower, healthier PWV. These findings are in agreement with other previously conducted observational studies in healthy participants which have shown an inverse association between vitamin D and arterial stiffness (Al Mheid *et al.* 2011; Giallauria *et al.* 2012; Pirro *et al.* 2012; Seker *et al.* 2013; Webb *et al.* 2012). The largest of these recruited 1228 healthy adults aged 70 ± 12 y and measured PWV using the Complior SP device (Artech Medical, Paris, France) (Giallauria *et al.* 2012). After adjustment for age, gender, race, season of blood draw, and PTH levels, PWV remained significantly associated with serum 25-OH-D (β=-0.43, P=0.001) (Giallauria *et al.* 2012). Similarly, after multivariate adjustment for a number

of factors including age, race, gender, BMI and lipids, serum 25-OH-D remained independently associated with PWV measured using the SphygmoCor device (adjusted R^2=0.32, β=-0.09, *P*=0.04) in a study of 554 healthy adults aged 20 to 79 y (Al Mheid *et al.* 2011). Another study in 150 healthy postmenopausal women that also used SphygmoCor to measure PWV initially found a significant inverse association with 25-OH-D concentrations (*ρ*=-0.23, *P*=0.006), but this disappeared after controlling for PTH (Pirro *et al.* 2012). Clinical trials of the effect of vitamin D supplementation on PWV are few in number, and mostly in participants with diseases including type 2 diabetes (Yiu *et al.* 2013; Breslavsky *et al.* 2013) and Peripheral Arterial Disease (Stricker *et al.* 2012). They have presented mixed results, although most show no effect (Gepner *et al.* 2012; Stricker *et al.* 2012; Yiu *et al.* 2013; Larsen *et al.* 2012). There is speculation that vitamin D may have a positive effect on arterial stiffness via a number of mechanisms including inhibition of the renin-angiotensin system (Li *et al.* 2002; Li 2003), protection against vascular calcification (Watson *et al.* 1997) or suppression of vascular smooth muscle cell proliferation (Chen *et al.* 2010). However, as the data used in the present analysis were cross-sectional, the results for PWV do not prove causality and it may be that people who go outdoors more often who are exposed to a higher level of UVB radiation do more physical activity which may be having favourable effects on arterial stiffness (Sugawara *et al.* 2006). Whilst a significant inverse correlation was found between serum 25-OH-D concentrations and 24 h DBP (*ρ* =-0.10, *P*=0.03), a comparison of 24 h DBP between different categories of vitamin D status did not quite reach significance (*P*=0.06), although there was a trend for it to decrease with increasing serum 25-OH-D. Many other previous cross-sectional studies have investigated the association between vitamin D status and BP, with most showing an inverse association for both SBP and DBP (Judd *et al.* 2008; Schmitz *et al.* 2009; Scragg *et al.* 2007; Zhao *et al.* 2010; Scragg *et al.* 1920). However, the majority of studies have used clinic BP rather than ABP, which is a more accurate measure (Coats *et al.* 1992). Trials investigating the effect of vitamin D supplementation on BP have also been conducted and summarised in meta-analyses (Kunutsor *et al.* 2014; Pittas *et al.* 2010; Elamin *et al.* 2011; Witham *et al.* 2009; Wu *et al.* 2010), but the results are inconsistent, and again, all or nearly all studies included have used clinic BP. There is a need for well-conducted trials measuring BP using ABP monitors, and PWV to help

determine whether the associations are causal and vitamin D supplementation can improve these measures.

FMD was not significantly correlated with serum 25-OH-D concentrations, but there was a significant difference in vitamin D status between participants with serum 25-OH-D concentrations of 50-75 nmol/L and those with concentrations more than 75 nmol/L, with participants in the higher category having improved endothelial function. Although there were only small numbers in the +75 nmol/L group (*n*=38), this association remained after adjustment for age, gender, BMI, ethnicity, physical activity and seasonality. Other cross-sectional studies have mostly shown positive associations between 25-OH-D concentrations and FMD (Al Mheid *et al.* 2011; Jablonski *et al.* 2011; Syal *et al.* 2012; Yiu *et al.* 2011). RCTs, however, have shown mixed results but many, in accordance with the present study, show no positive relationship between vitamin D and FMD (Gepner *et al.* 2012; Witham *et al.* 2010; Yiu *et al.* 2013).

The inflammatory marker hsCRP was inversely correlated with vitamin D status for both studies combined (*P*=0.01) and there was a trend for concentrations to decrease as the serum 25-OH-D category increased, although this did not reach significance. In the vitamin D deficient group (serum 25-OH-D <25 nmol/L) hsCRP concentrations were high at 2.3 mg/L. The literature has mixed findings on the relationship between 25-OH-D and hsCRP. In agreement with the present study, Amer *et al.* analysed NHANES data from between 2001 to 2006 and showed a significant inverse association between serum 25-OH-D concentrations and CRP for 25-OH-D concentrations < 52 nmol/L (*n* > 17,000). Interestingly, however, above 52 nmol/L, the relationship was positive (Amer and Qayyum 2012). In the present study, serum 25-OH-D concentrations continued to decrease between the categories 50-75 nmol/L and 75+ nmol/L for both studies combined. Another study by Ngo *et al.* found an inverse association between hsCRP concentrations and serum 25-OH-D_3 in 253 adults aged 51-77 y after adjustment for age, gender, hypertension and BMI (β=-0.14, *P*=0.03) (Ngo *et al.* 2010). However, other studies found the initial association between vitamin D and hsCRP to disappear after adjustment for adiposity (Hypponen *et al.* 2010) or BMI (Jorde *et al.* 2007), or did not find any association at all (Michos *et al.* 2009). Mechanisms as to how vitamin D may affect CRP levels and reduce the inflammatory response are uncertain, but

experimental studies have shown that treatment with $1,25(OH)_2D$ inhibits the production of several other pro-inflammatory cytokines such as interleukin-6 (IL-6) (Talmor *et al.* 2008; Muller *et al.* 1992) and tumour necrosis factor-α (TNF-α) (Muller *et al.* 1992; Cohen-Lahav *et al.* 2007).

Strengths of the analyses conducted in this chapter are that they included a large number of participants who were healthy without disease-related complications, and a range of CVD risk markers. Furthermore, carotid-femoral PWV and ABP were measured which are considered to be the 'gold-standard' measurements for arterial stiffness (Shahin *et al.* 2013) and BP (National Institute for Health and Clinical Excellence 2011), respectively. Additionally, adjustments were made for a number of potential confounding factors including age, BMI, gender, seasonality, physical activity and ethnicity. A limitation is that the study is cross-sectional and therefore causality cannot be assumed.

In conclusion, the above has shown that dietary intake is an important determinant of vitamin D status in this UK population, despite status being primarily dependent on the skin's exposure to UVB radiation (Zittermann *et al.* 2005), and that modifications to dietary intake, specifically increases in oily fish consumption, can significantly improve vitamin D status in healthy adults. Furthermore, the results strongly support the hypothesis that serum 25-OH-D concentrations are associated with arterial stiffness in healthy older adults without clinical disease, and provide evidence for potential associations with DBP and hsCRP as well. These findings provide a basis for an intervention trial to test the hypothesis that vitamin D supplementation improves markers of vascular function and inflammation.

Chapter 4

Comparison of the effect of low dose D_2 and D_3 in their capacity to raise serum 25-hydroxy-vitamin D concentrations

4.1 Introduction

The main purpose of this thesis is to determine the effect of dietary vitamin D on CVD risk. This can be done by comparing the effects of vitamin D_2 with placebo, enabling differentiation between the effects of diet and sunlight as D_2 increases serum 25-hydroxyvitamin-D_2 (25-OH-D_2), rather than 25-hydroxyvitamin D_3 (25-OH-D_3) which is produced in response to UVB exposure. However, it is first necessary to ascertain the extent to which D_2 increases serum 25-OH-D concentrations in the UK winter months, compared with vitamin D_3 as there has been some controversy surrounding the efficacy of D_2 (Houghton and Vieth 2006).

Pharmacopoeias regard D_2 and D_3 as equivalent and interchangeable with 1 international unit (IU) being equivalent to 25 ng of D_2 or D_3 (Medicines Commission 1980; Committee of Revision 1997). Furthermore, studies have shown the two forms to be equally effective in treating hypovitaminosis D in healthy and rachitic infants and children (Gordon *et al.* 2008; Thacher *et al.* 2010). However, Houghton and Vieth have argued that D_2 is less effective than D_3 at raising serum 25-OH-D concentrations, and have recommended that D_2 should no longer be regarded as suitable for supplementation (Houghton and Vieth 2006). This conclusion is questionable though as it was based on studies that did not use specific assays for measuring 25-OH-D metabolites (de la Hunty *et al.* 2010; Romagnoli *et al.* 2008; Trang *et al.* 1998; Heaney *et al.* 2011), had flaws in their design and execution (i.e. lacking a placebo treatment) (Trang *et al.* 1998; Romagnoli *et al.* 2008; Armas *et al.* 2004), did not control for UVB exposure (Binkley *et al.* 2011), or used very high doses of vitamin D that are not relevant to dietary intake (Romagnoli *et al.* 2008; Armas *et al.* 2004; Binkley *et al.* 2011; Heaney *et al.* 2011). Consequently, there is a need for a well-controlled, dose-response trial of food fortified with D_2 and D_3 using specific assays for 25-OH-D_2 and 25-OH-D_3 (O'Donnell *et al.* 2008). An intake of 5 µg represents the recommended daily amount for food labelling purposes in the European Union (European Union 2008), and 10 µg is the amount recommended in the UK for the house-bound who are unlikely to have much sun exposure (Scientific Advisory Committee on Nutrition 2007).

The aim of the present study was to evaluate the effect on serum 25-OH-D metabolites of intakes of 5 and 10 μg/d of D_2 and D_3 (5-D_2, 10-D_2, 5-D_3 and 10-D_3), provided in a malted milk drink, in the UK winter months when UVB exposure is minimal.

4.2 Methods

4.2.1 Hypothesis

Vitamins D_2 and D_3 provided at doses of 5 or 10 μg/d in a malted milk drink will result in meaningful increases in serum 25-OH-D_2 and 25-OH-D_3 respectively in healthy men and women with negligible UVB exposure.

4.2.2 Objective

To conduct a randomised controlled trial in healthy men and women to compare the effects on serum 25-OH-D concentrations of 5 or 10 μg/d D_2 or D_3 provided in a malted milk drink in the winter months in the UK.

Primary outcome:

Serum 25-OH-D metabolite concentrations.

Secondary outcome:

Parathyroid hormone (PTH) and serum calcium (Ca^{2+}) concentrations.

4.2.3 Sample size

Sample size calculations were based on a pilot study conducted in 6 healthy volunteers recruited in the month of February in which the mean plasma 25-OH-D concentrations as measured by RIA increased from 60 nmol/L, to 70, 78 and 90 nmol/L after 1, 2 and 3 wk of supplementation with 10 μg D_3 in cod-liver oil (2 capsules/d, each containing 5 μg as specified by the manufacturer, Seven Seas Lt, Hull). Repeated measures on unsupplemented participants showed a correlation of 0.91 and the common standard deviation (SD) was 17 nmol/L. The power calculation for the present study performed in STATA version 9.0 is shown below. Assuming at least two measurements on follow up allowed division of the SD by √2 (17/√2 = 12.0). A sample size of 8 per treatment group with a minimum of two measures on follow up was estimated to be able to detect a 10 nmol/L change, which was deemed to be clinically significant, in serum

vitamin D concentrations with α=0.01 (to allow for multiple comparisons) and 90% power. Additional power is gained for the comparison of D_2 with D_3 if both 5 and 10 μg/d doses are compared with 16/group giving power to detect differences of 5 nmol/L between the combined groups.

Stata syntax:

power pairedmeans, corr(0.91) altdiff (10) power (0.9) sd (12) alpha (0.01)

Stata output:

Estimated sample size for a two-sample paired-means test

Paired t test assuming sd1 = sd2 = sd

Ho: d = d0 versus Ha: d != d0

Study parameters: alpha = 0.0100, sd = 12.0000, power = 0.9000, corr = 0.9100

delta = 1.9642, d0 = 0.0000, da = 10.0000 sd_d = 5.0912

Estimated sample size: N = 8

4.2.4 Ethical approval and clinical governance

Ethical approval was obtained from the South East London Research Ethics Committee 1 (REC reference 10/H0804/91) on 07/02/2011 (**Appendix 10**). The trial was registered at http://www.controlled-trials.com and allocated a clinical trial registration number ISRCTN24666304 on 02/08/2011. Written informed consent (**Appendix 11**) was obtained from all participants before commencing the study and phlebotomy was conducted by appropriately qualified staff.

4.2.5 Study design

A placebo controlled double-blind parallel design was used. Participants were randomly allocated by computer to one of 5 groups: placebo or 5 or 10 μg of D_2 or D_3. The intervention was administered as a malted milk drink (Horlicks™, GlaxoSmithKline, Middlesex, UK).

The study took place from 22/02/2011 – 13/04/2011, a period during which there is usually minimal UVB exposure in the UK (**Appendix 12**). Following screening, eligible participants were asked to attend the Metabolic Research Unit (MRU) in the Diabetes and Nutritional Sciences Division at KCL for 5 visits (baseline and every week after this

for 4 wk). They were also sent a FFQ used in the European Prospective Investigation into Cancer and Nutrition (EPIC) to determine their usual vitamin D intake (**Appendix 9**). This FFQ has been validated for vitamin D intake in the UK population (Crowe *et al.*). They were requested to fill this in at home and return it at their baseline visit at which each participant was given 28 sachets each containing 25 g of malted milk drink. They were asked to start drinking a sachet a day, made up with hot water, for 28 days, starting on the day of the visit. It was stressed that they should only add hot water, and not milk, to try and minimise any changes in calcium intake. At each visit, a blood sample was collected by a trained phlebotomist according to the blood collection protocol outlined in **Appendix 6**. The vacutainer method (Becton-Dickinson) was used to reduce the risk of spillages. At baseline and visit 4, the blood sample was analysed for PTH, Ca, 25-OH-D_2 and 25-OH-D_3. Blood samples taken at wk 1, 2 and 3 were analysed for serum 25-OH-D_2 and 25-OH-D_3 only (**Figure 4.1**).

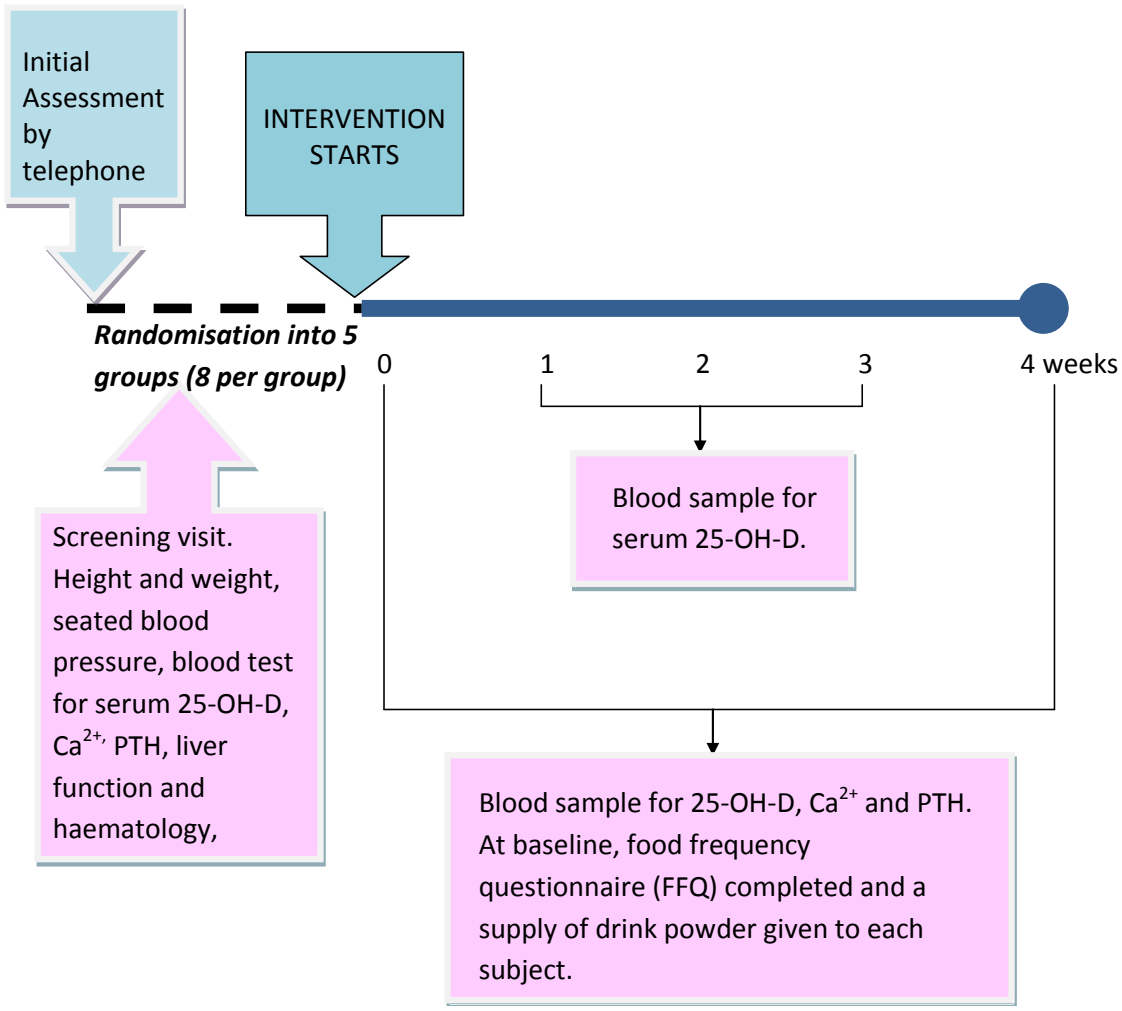


Figure 4.1 Study Design

4.2.6 Recruitment methodology

Participants (*n*=40) were recruited from the staff and student population of KCL staff and students. They were initially contacted via an ethically approved email circular (**Appendix 13**). Subjects expressing interest were provided with a study information sheet (**Appendix 14**) and asked to complete a telephone/email questionnaire (participants had the option as to whether to complete the questionnaire sent by email or to ring up and complete it over the telephone) if still interested (**Appendix 15**). Those who appeared eligible and willing to participate were invited to attend a screening appointment at the Franklin-Wilkins Building in the MRU to further assess their suitability for inclusion in the study. BP and anthropometric measurements were taken, along with a blood sample to measure plasma PTH, serum Ca^{2+} and 25-OH-D and ascertain that haematology, liver function and plasma glucose concentrations

were within prescribed limits (**Appendix 16**). A flow chart detailing the process is show in **Figure 4.2**.

Inclusion criteria were healthy males or females aged 18-65 y with normal haematology, liver function and plasma glucose. Exclusion criteria were: seated BP >160/105 mm Hg, Body Mass Index <18.5 and >35 kg/m^2, taking vitamin and mineral supplements (including cod-liver oil), or prescription calcium/vitamin D, recent exposure to high UVB light since 1 Dec 2010, intolerance to study product (lactose, milk protein), chronic renal, liver or inflammatory bowel disease, diabetes, unwillingness to restrict consumption of oily fish to no more than 2 portions (98 g/portion)/wk throughout the study, and unwillingness to abstain from nutritional supplements for the duration of the study.

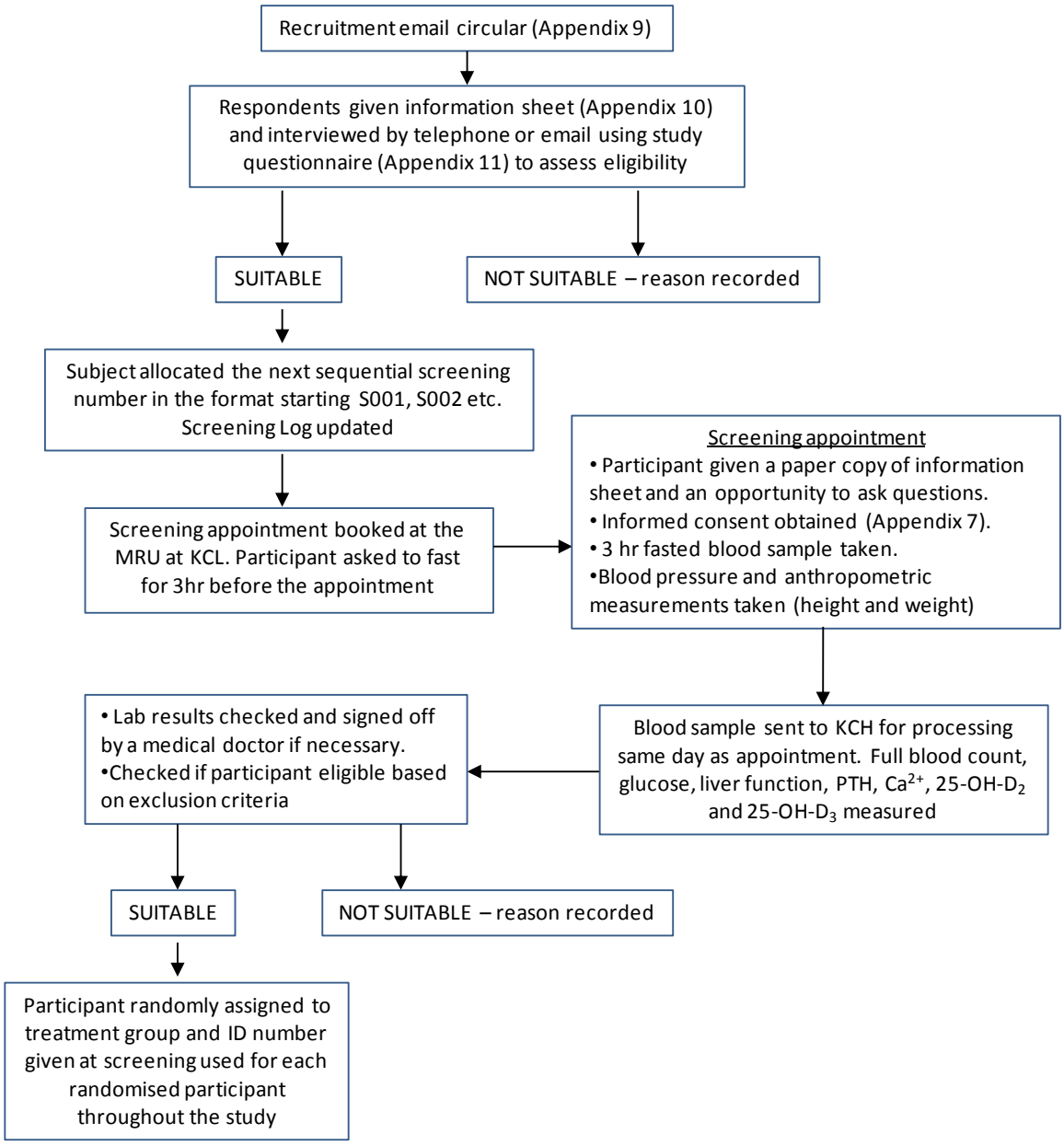


Figure 4.2 Recruitment, screening and randomisation

4.2.7 Habitual dietary intake analysis

FFQs were analysed using programs created in Microsoft Access and Excel for use in previous studies carried out at KCL. These programs had originally been set up to determine dietary intake per day for each participant for the macronutrients. In order for the program to be able to calculate vitamin D values for each food in the FFQ, vitamin D values in µg per 100 g were added to the database. These values were determined from supermarket websites (for example, for specific cereals), McCance and Widdowson's "The Composition of Foods: Summary Edition (6th Edition)"

(McCance and Widdowson 2002), and the USDA National Nutrient Database for Standard Reference (USDA 2014). The FFQ asked about the average frequency of consumption of 130 food and beverage items in the preceding 12 months with 9 frequency options (**Appendix 9**). If a participant had changed their diet in the last few months, they were asked to answer the FFQ questions based on their most recent diet. Average portion sizes had previously been derived for each of the food and beverage items based on those used originally in the EPIC questionnaire, values in "Food Portion Sizes" by the Ministry of Agriculture, Fisheries and Food (Crawley 1994), and "A Photographic Atlas of Food Portion Sizes" by the Ministry of Agriculture, Fisheries and Food (Nelson 1997). Portion fractions had also previously been decided for each of the frequency options. Nutrient intake per day was calculated based on the intake of the 130 food and beverage items recorded by each participant. The database was set up to calculate macronutrients based on a default cereal (Cornflakes). In order to obtain accurate vitamin D estimates for cereal consumption, the vitamin D content of the specific cereals entered was determined separately and added on to the total vitamin D intake per day for each participant where relevant. Calculations were also performed to determine the macronutrient (energy, fat, carbohydrate and protein) and vitamin D contribution from extra foods for the participants who had entered these, and values were added to the total intakes per day.

4.2.8 Sample analysis

Table 4.1 shows the methods used and where the analyses were conducted, and gives reference to the appropriate section in Chapter 2 where the methods are described in detail.

Table 4.1 Biochemical measurements of study day samples

Analyte	Method	Section where details can be viewed	Where analysis was conducted
PTH	Immunoassay	2.4.7	KingsPath
Calcium	Colourimetric assay	2.4.5	KingsPath
25-OH-D_2 and 25-OH-D_3	uHPLC-MS/MS	2.4.6.1	LGC/HFL Sport Science

PTH, parathyroid hormone; KingsPath, King's College Hospital; LGC, LGC Testing (Formerly HFL Sports Science, Cambridgeshire); uHPLC-MS/MS, ultra high pressure liquid chromatography tandem mass spectrometry.

4.2.9 Composition of malted milk drink

Each 25 g sachet provided: 92 kcal, 2.4 g protein, 18.9 g carbohydrate (of which sugars, 9 g), 0.8 g fat (of which saturates, 0.3 g) and 100 mg sodium. Prior to the start of the study, the D_2 and D_3 content of the malted milk drink was analysed by International Food Network (IFL) using an accredited method based on 100°C + KOH extraction, solid phase extraction to remove interfering components, and HPLC (**Appendix 17**). The limit of detection for this method was 0.5 µg/100 g. This analysis confirmed no vitamin D_2 or D_3 in the placebo sample. The vitamin D_3 levels were found to be within 0% and 10% of the target amounts of 5 µg and 10 µg respectively (5.2 µg for the 5 µg sachets and 10 µg for the 10 µg sachets). However, the analysis showed an insufficient amount of D_2 in the samples and therefore an additional analysis was requested from the Laboratory of Government Chemists PLC (Teddington, UK). This could have been due to inherent analysis uncertainties which are +/- 20% and +/- 10% respectively. It could also be due to the lab not being able to extract all of the vitamin D_2 from the food matrix (the extraction phase sometimes causes issues in products that are encapsulated, as the vitamin D_2 was). The analysis at LGC (**Appendix 17**) used the same method as described above, with a limit of detection of 0.1 µg/100 g, and found much improved values for vitamin D_2 (4.8 µg for the 5 µg sachets and 7.5 µg for the 10 µg sachets). However, the values for vitamin D_3 were about half of what they should have been. It is possible that this could be due to a sampling error as a sample was taken from a pool of 3 samples. Vitamin D is microencapsulated in the powder so it is possible for the distribution not to be uniform in a small sample taken for analysis.

4.2.10 Statistical analyses

Statistical analysis of the data was conducted using SPSS for Windows Version 20.0. Where values for 25-OH-D_2 were below the limit of detection (2.4 nmol/L), a value of 2.4 nmol/L was assigned. Standard distributional checks were made, and where appropriate, analyses were attempted following log transformation. Data were analysed on an intention to treat basis by repeated measures analysis of variance (4 time points) with the baseline value as a covariate and treatment group as the between-subjects factor. Repeated measures contrasts were performed on the data to test for a dose response and compare the 5 and 10 µg groups in the changes in 25-OH-D metabolite concentrations from baseline to post-intervention. The incremental area under the curve (iAUC) was calculated using the trapezoid rule. Comparison of the iAUC and changes at wk 4 from baseline were compared by one-way analysis of variance and Dunnett's test was used to indicate a significant difference compared with placebo. All values in the text are mean ± SD unless otherwise stated.

4.3 Results

4.3.1 Participant details

Forty participants (*n* = 16 males, *n* = 24 females) were randomly and equally allocated into the 5 treatment groups and the flow of participants through the study is detailed in **Figure 4.3**.

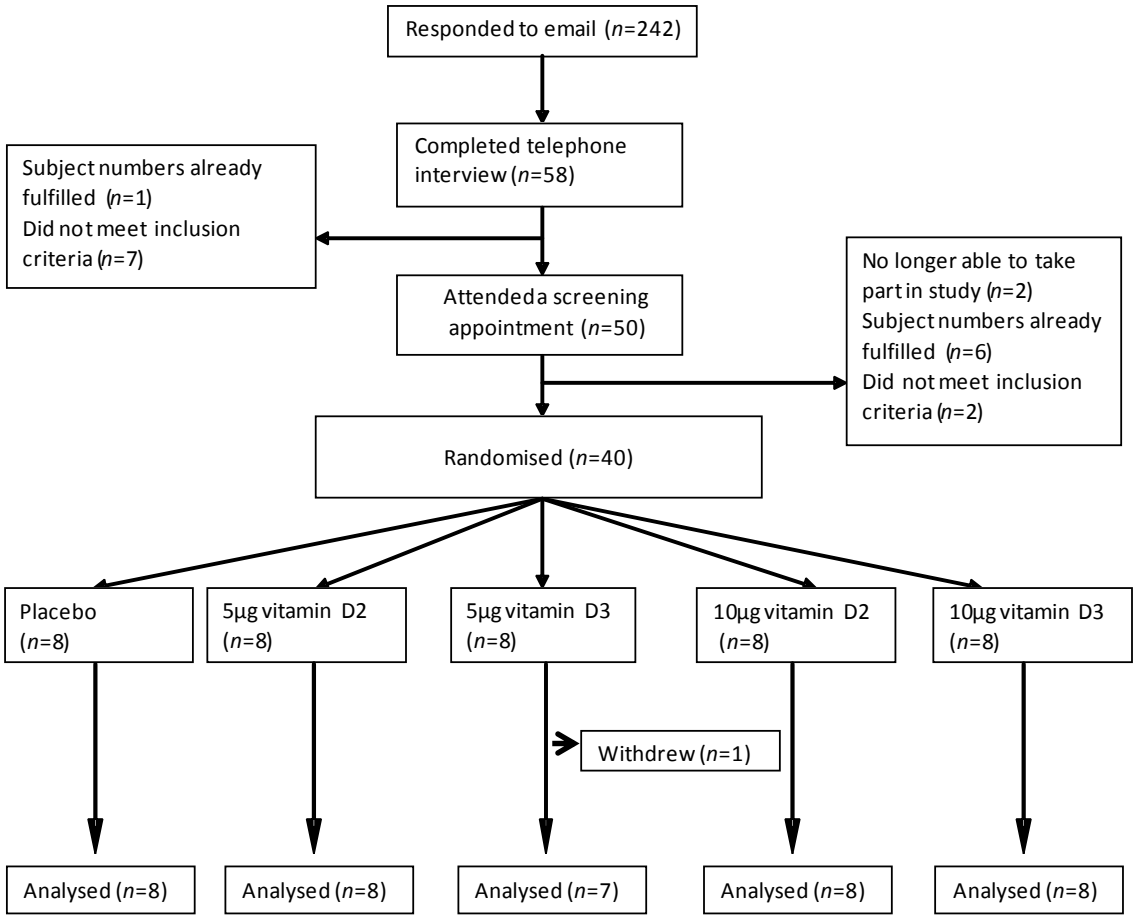


Figure 4.3 Flow of participants through the study

One female disliked the test drink and withdrew from the study. Data were available for analysis on 39 participants, who reported consuming all 28 sachets. Baseline characteristics are displayed in **Table 4.2**. There were no significant differences across treatment groups in age, height, weight, BP, vitamin D intake as assessed by FFQ, 25-OH-D concentrations, PTH levels, or Ca^{2+} concentrations. The BMI in participants in the 5-D_3 group was slightly higher than in the 5-D_2 group. Mean baseline concentrations of 25-OH-D_2, 25-OH-D_3 and total 25-OH-D were 3, 38 and 42 nmol/L, respectively. A total of 27.5, 67.5 and 90.0% of participants were classified as being below the cut-offs of 25 nmol/L, 50 nmol/L and 75 nmol/L, respectively.

Table 4.2 **Baseline characteristics of participants at randomisation by treatment allocation (*n*=40)[1, 4]**

Characteristics	Placebo	5-D_2	5-D_3	10-D_2	10-D_3
Male: female	2:6	3:5	3:5	3:5	5:3
Age, *y*	24.1 ± 1.8	24.4 ± 4.7	30.5 ± 11.0	24.4 ± 3.9	30.6 ± 10.6
White: non-white	6:2	6:2	6:2	8:0	5:3
Height, cm	170.1 ± 7.3	175.8 ± 8.1	171.9 ± 9.4	170.1 ± 7.9	169.5 ± 10.7
Weight, kg	61.8 ± 9.8	63.0 ± 7.3	74.7 ± 11.6	66.7 ± 11.3	71.9 ± 17.1
BMI, kg/m^2	21.3 ±2.1	20.4 ±1.5	25.7 ±2.8	22.9 ±2.7	24.8 ±3.9
Systolic/diastolic BP (mm Hg)	113/71 ± 11/5	118/77 ± 6/8	119/77 ± 12/4	116/74 ± 9/4	127/78 ± 17/10
Vitamin D intake,[2],*µg/d*	2.4 ± 1.6	2.6 ± 1.3	2.4 ± 1.3	3.2 ± 1.3	2.6 ± 1.2
Calcium intake, mg/d	902 ±346	806 ±223	880 ± 249	1000 ±220	1019 ± 279
Serum 25-OH-D_2, nmol/L	3.4 ± 1.2	5.9 ± 8.2	2.7 ±0.7	2.8 ± 0.6	2.5 ± 0.1
Serum 25-OH-D_3, nmol/L	32.3 ± 13.7	49.4 ± 27.6	34.4 ± 22.1	41.3 ± 14.1	34.9 ± 29.1
Total serum 25-OH-D, nmol/L	35.6 ± 13.3	55.3 ± 26.6	37.1 ± 22.1	44.0 ± 14.1	37.3 ± 29.1
Serum Ca^{2+},[3] mmol/L	2.25 ± 0.07	2.31 ± 0.05	2.28 ± 0.05	2.27 ± 0.07	2.25 ± 0.07
Plasma PTH,[3], ng/L	48.0 ± 10.1	44.9 ± 9.2	40.9 ± 11.7	45.7 ± 9.9	47.0 ± 15.1

[1]Values are means ± SD. 5-D_2, 5 µg ergocalciferol/d; 5-D_3, 5 µg cholecalciferol/d; 10-D_2, 10 µg ergocalciferol/d; 10-D_3, 10 µg cholecalciferol/d; PTH, parathyroid hormone.

[2]Includes both D_2 (ergocalciferol) and D_3 (cholecalciferol) from fortified foods.

[3]Values are geometric means ± SD.

[4]No statistically significant differences between groups at baseline assessed by chi-squared test, Kruskall-Wallis test, or one-way analysis of variance except for BMI which was significantly greater in participants in the 5-D_3 group compared to those in the 5-D_2 and in the 10-D_3 group compared with the 5-D_2 group (Bonferroni Multiple comparison $P<0.05$)

4.3.2 25-OH-D

Raw data is displayed in **Appendix 18**. Serum vitamin D metabolite concentrations remained low and unchanged in the placebo group. The 25-OH metabolite concentrations rose steadily in the first three weeks of supplementation and appeared to have reached a plateau at 4 wk as there was no evidence of further increases between weeks 3 and 4 (**Figure 4.4**). There was a significant dose-response relationship between the changes in plasma analyte concentrations. Repeated measures contrasts showed greater increases in 25-OH-D_2 over the 4 wk in the 10-D_2 group compared to the 5-D_2 group (P = 0.007). Similarly, the increase in 25-OH-D_3 in the 10-D_3 group was greater than that in the 5-D_3 group (P = 0.003) and the increase in total 25-OH-D in the two 10 µg/d groups was greater than that in the two 5 µg/d groups (P=0.01). Vitamin D_2 did not affect the concentration of 25-OH-D_3, nor did D_3 affect that of 25-OH-D_2. Significant differences versus placebo were found in total 25-OH-D for 5-D_2, 10-D_2, 5-D_3 and 10-D_3 treatment groups (P≤0.001). Compared with placebo, the increment in serum 25-OH-D_2 from baseline to post-intervention was greater in the 5-D_2 and 10-D_2 groups (both P<0.01), as was the increase in 25-OH-D_3 greater in the 5-D_3 and 10-D_3 groups (P<0.05 and P<0.01) (**Table 4.3**). The increase in 25-OH-D_2 in the D_2 groups was not significantly different to the increase in 25-OH-D_3 for the D_3 groups (iAUC 24 ± 11, 45 ± 26, 23 ± 13 and 49 ± 25 nmol·L^{-1}·wk for 5-D_2, 10-D_2, 5-D_3 and 10-D_3). Furthermore, the increment in serum total 25-OH-D did not differ between D_2 and D_3 supplement groups (P=0.945) (**Figure 4.4**) and the iAUCs for total 25-OH-D in the D_2 groups tended to be lower but did not differ significantly from those in the D_3 groups (iAUC 15 ± 15, 35 ± 27, 23 ± 14 and 49 ± 24 nmol·L^{-1}·wk for 5-D_2, 10-D_2, 5-D_3 and 10-D_3 respectively).

The analyses were also conducted adjusting for gender, age and BMI. There were no significant gender or BMI effects or interactions, but there was a significant age x time interaction (P = 0.037).

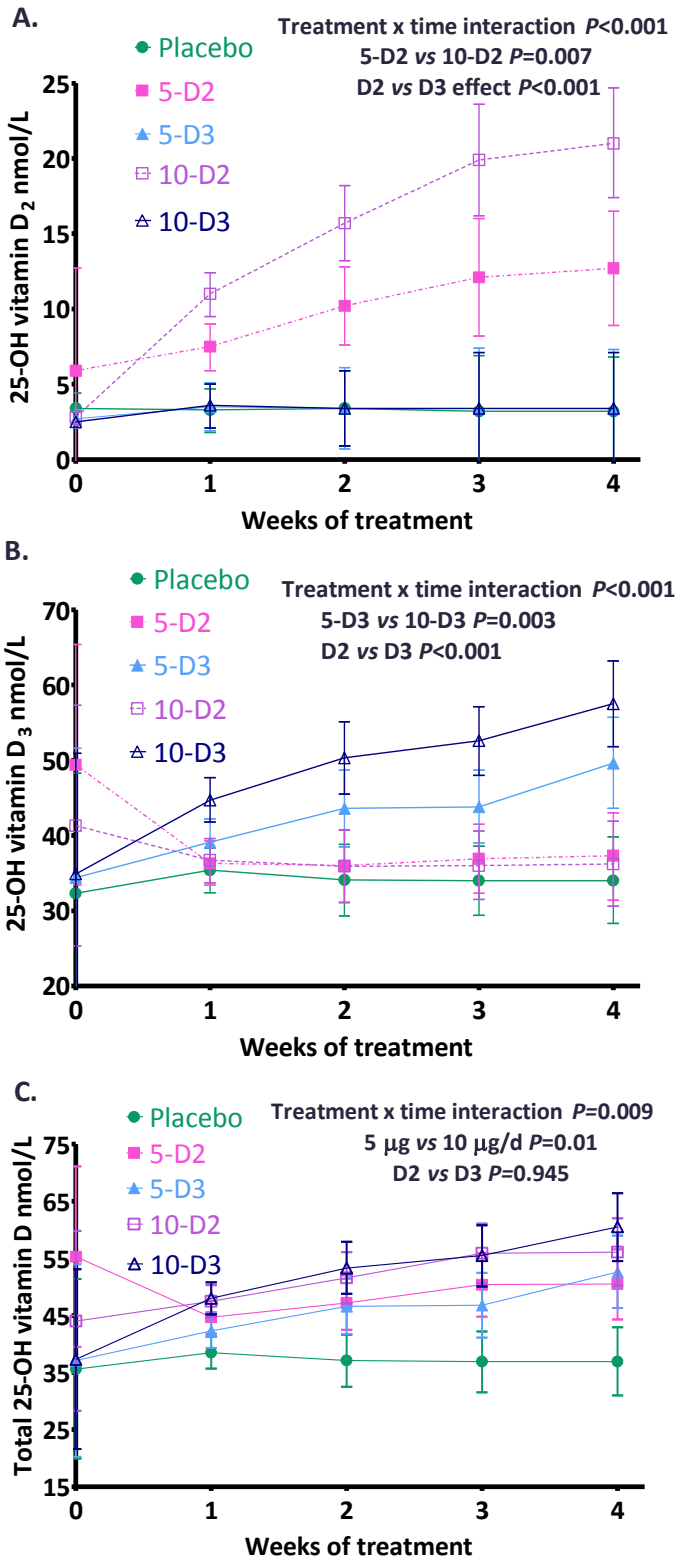


Figure 4.4 Serum concentrations of 25-OH-D_2 (panel A), 25-OH-D_3 (panel B) and total 25-OH-D (panel C) in healthy men and women not exposed to UVB following placebo (*n*=8), 5 µg ergocalciferol/d (5-D2), *n*=8; 5 µg cholecalciferol/d (5-D3), *n*=7; 10 µg ergocalciferol/d (10-D2), *n*=8 and 10 µg cholecalciferol/d (10-D3), *n*=8 for 4 wk in the winter months. Values are means with 95% CI. Data were analysed by repeated measures analysis of variance (4 time points) with the baseline value as a covariate and treatment group as the between-subjects factor. Repeated measures contrasts were performed to test for a dose response effect.

Table 4.3 Changes (Δ) in serum 25-OH-D metabolite, serum calcium and plasma parathyroid hormone (PTH) concentrations after 4 wk of consuming a drink providing a daily intake of 5 μg or 10 μg of ergocalciferol (D_2) or cholecalciferol (D_3) or placebo in participants not exposed to UVB[1].

Analyte	Placebo	5-D_2	5-D_3	10-D_2	10-D_3	*P* value[2]
Δ25-OH-D_2, nmol/L	-0.2 (-1.0, 0.5)	9.2 (5.5, 12.9)**	-0.1 (-0.2, 0.1)	17.6 (9.2, 25.9)**	- 0 (-0.1, 0.1)	<0.0001
Δ25-OH-D_3, nmol/L	-3.1 (-8.0, 1.7)	-3.8 (-10.3, 2.8)	12.0 (2.7, 21.2)*	-2.9 (-9.3, 3.4)	19.8 (9.4, 30.2)**	<0.0001
ΔTotal 25-OH-D, nmol/L	-3.4 (-8.2, 1.5)	4.9 (-2.3, 12.7)	11.9 (2.7, 21.2)*	13.6 (4.1, 23.0)**	19.7 (9.4, 30.1)**	0.001
ΔCa^{2+}, μmol/L	-23 (-54, 99)	-39 (-145, 67)	-17 (-87, 57)	-15 (-85, 123)	-21 (-85, 124)	0.728
ΔPTH, ng/L	-6.6 (-22.0, 9.7)	3.8 (-18.1, 25.7)	2.4 (-20.0, 24.9)	-10.6 (-10.0,-1.2)	-15.7 (-15.7, 2.6)	0.269

[1] Values are mean changes from baseline with 95% CI, *n*=8 for every group apart from the 5-D_3 group for which *n*=7. 5-D_2, 5 μg ergocalciferol/d; 5-D_3, 5 μg cholecalciferol/d; 10-D_2, 10 μg ergocalciferol/d; 10-D_3, 10 μg cholecalciferol/d; PTH, parathyroid hormone.

[2] Probability from oneway ANOVA of the changes from baseline between treatment groups.

**P*<0.05, ***P*<0.01 compared with placebo; Dunnett's test.

4.3.3 Calcium

There was no significant difference in the change in serum Ca^{2+} concentrations from baseline to post-intervention between treatment groups (**Table 4.3**).

4.3.4 PTH

Although there was a decrease in PTH of 13 ng/L (95% CI: -21, -3) in the 10 µg/d groups compared to baseline, this change was not significantly different from the change in the placebo group (**Table 4.3**).

4.4 Discussion and conclusion

This study set out to compare the effects of intakes of 5 or 10 µg/d D_2 or D_3, as might be provided by fortified foods, on serum vitamin D metabolite concentrations. It addresses an important question regarding the efficacy of fortified foods in helping maintain normal serum 25-OH-D concentrations. At baseline, 67.5% of participants had a 25-OH-D concentration below 50 nmol/L. This level is currently generally regarded as representing the lower limit of adequacy in vitamin D status. After a month of 5 or 10 µg/d of D_2 or D_3, only 35% of participants in the treatment groups still had values below 50 nmol/L. The study is particularly relevant to the UK because of the relatively large population of vegetarians, particularly those of South Asian origin. D_2 is derived from fungi and is acceptable to people unwilling to consume animal products, whereas D_3 is derived from animal products. This study was conducted at a time of year when UVB exposure is extremely limited in the UK. The precaution of excluding participants who had recent high exposure to UVB was also taken. Most participants did not consume any D_2. However, in one participant, high (>25 nmol/L) serum concentrations of 25-OH-D_2 were found at baseline that were later found to be derived from regular consumption of fortified soya milk. Nevertheless, a very clear dose response relationship between D_2 intake and serum 25-OH-D_2 was shown, and there was considerably less variability in this analyte than that of 25-OH-D_3 which is influenced by UVB exposure as well as dietary intake. There was no evidence of any change in 25-OH-D_3 concentrations in the participants allocated to placebo or the D_2 groups confirming that the results were not confounded by variations in exposure to UVB during the study.

Both forms of vitamin D resulted in significant increments in their respective 25-OH-D metabolites compared with placebo, but the size of the increment did not differ between D_2 and D_3 treatments. There was a trend for the increment in 25-OH-D_3 to be greater than that of 25-OH-D_2, but there was greater variability in this compared to 25-OH-D_2, as shown by the larger confidence intervals. Furthermore, the increment in 25-OH-D_2 was reduced by the use of the limit of detection. Although four measurements were obtained on treatment for every participant compared to the minimum of 2 on follow-up required for the sample size calculation, and the comparison between D_2 and D_3 included 16 participants in each group, rather than 8; both factors which allow for the detection of a smaller difference than 10 nmol/L, the sample size used in this study may be insufficient to detect more subtle differences between D_2 and D_3. Before any definitive conclusion can be drawn on the equivalence of D_2 and D_3, a larger sample size with a more diverse population would be required, particularly including those with an elevated BMI (>30 kg/m^2).

Much of the uncertainty regarding the ability of the two forms to increase vitamin D status has arisen because of the use of non-specific assays. Several previous studies have concluded that D_2 is less effective than D_3 at increasing serum 25-OH-D concentrations (Armas *et al.* 2004; Trang *et al.* 1998; Romagnoli *et al.* 2008; Binkley *et al.* 2011; Heaney *et al.* 2011), but these were all uncontrolled, and three used an assay that was not able to measure 25-OH-D_2 and 25-OH-D_3 separately (Heaney *et al.* 2011; Romagnoli *et al.* 2008; Trang *et al.* 1998). All of the studies, apart from the study by Binkley *et al.* (Binkley *et al.* 2011), which gave participants 40 µg/d or 1250 µg/wk, also used supplements providing more than 100 µg. Two gave very large single doses of vitamin D at 7500 µg (Romagnoli *et al.* 2008) or 1250 µg (Armas *et al.* 2004). It is not possible to extrapolate from such high doses to the low amounts provided in fortified foods.

A strength of this study is that a highly sensitive UPLC-MS/MS assay was used which separated the two vitamin D metabolites. This assay only uses 0.1 mL of serum but has sensitivity over the range 2.4 to 363.5 nmol/L for 25-OH-D_2, and 7.5 to 374.4 nmol/L for 25-OH-D_3, with accuracies of between 0.5% and 6.6% for both metabolites. This study compares the two forms of vitamin D at an intake within the dietary range and in

line with recommendations in healthy adults. The findings are broadly in agreement with a study by Holick *et al.* (Holick *et al.* 2008) in which healthy adults were randomly allocated to receive 25 µg of D_3 (*n*=20), 25 µg of D_2 (*n*=16), 12.5 µg of each of D_3 and D_2 (*n*=18), or placebo once a day for 11 wk. Their measurements were conducted using LC-MS/MS; the detection limit for the assay was 10 nmol/L and the interassay coefficient of variation was about 10%. They found both 25-OH-D_2 and 25-OH-D_3 to be equally effective. In our study, serum 25-OH metabolite concentrations appeared to plateau at 4 wk whereas in the study by Holick *et al*. it was suggested that the plateau was reached after 6 wk (Holick *et al.* 2008). However, closer inspection of their data suggests that later values fell back closer to the 4 wk value. A recent study by Biancuzzo *et al.* gave participants 25 µg/d of D_2 or D_3 in orange juice for 11 wk at the end of winter in the US. They similarly showed that a plateau in 25-OH-D_2 and 25-OH-D_3 was reached after 4-5 wk of supplementation, and that both D_2 and D_3 were equally effective at raising and maintaining 25-OH-D concentrations (Biancuzzo *et al.* 2010).

The mean absolute increases in the present study compared with placebo for 25-OH-D_2 were 9.4±2.5 and 17.8±2.4 nmol/L following 5-D_2/d and 10-D_2/d, and for 25-OH-D_3 were 15.1±4.7 and 22.9±4.6 nmol/L following 5-D_3/d and 10-D_3/d. This would suggest that for every 1 µg D_2 or D_3 there is about a 2 nmol/L increase in the 25-OH-D metabolite. For comparison, Holick *et al.* (Holick *et al.* 2008) found that supplementation with 25 µg/d led to serum 25-OH-D increases of 24.7 nmol/L in the D_2 group (*n*=16) and 23.2 nmol/L in the D_3 group (*n*=20) over 11 wk; equivalent to increases in serum 25-OH-D of approximately 1 nmol/L per µg vitamin D which is about half the value observed in the present study. It is possible that higher intakes become less effective at raising 25-OH-D concentrations because they increase the catabolic rate of the vitamin. However, the mean baseline 25-OH-D value in Holick's study was 47.0 nmol/L (Holick *et al.* 2008), compared to the mean of 42.0 nmol/L at baseline in this study and it may be that a greater treatment response is seen in participants who start with a lower serum 25-OH-D concentration.

Supplementation with D_2 did not influence serum concentrations of 25-OH-D_3. Previously, Armas *et al.* (Armas *et al.* 2004) and Thacher *et al.* (Thacher *et al.* 2010) have reported that a single 1.25 mg oral dose of vitamin D_2 led to a fall in 25-OH-D_3 in

adult men and healthy and rachitic Nigerian children, respectively. This may be due to competition for the 25-hydroxylase by D_2, an increased catabolism of 25-OH-D_3 and/or competitive displacement of 25-OH-D_3 from the VDBP by 25-OH-D_2. However, the findings in the current study support those of Holick *et al.* (Holick *et al.* 2008), and provide no evidence to suggest that D_2 influences the catabolism of D_3 or its metabolites. It may be only very high doses of vitamin D_2 that lead to strong enough effects on the factors listed above to cause a decrease in serum 25-OH-D_3 concentrations.

There was some tentative evidence for a decline in PTH concentrations with 10 µg/d of vitamin D but this was not significantly different compared with placebo. However as there is high variability between participants in PTH, it would be necessary to study a much larger number of participants to confirm this effect. It is also possible that due to the adequate calcium intakes at baseline, higher concentrations of serum 25-OH-D may not decrease PTH concentrations any further.

The strengths of this study are that it was conducted in the winter months using a physiologically relevant dose of vitamin D, and a highly sensitive and specific assay to measure 25-OH-D_2 and 25-OH-D_3. The food matrix used was a drink which may have facilitated absorption of the vitamin and so the results cannot necessarily be extrapolated to other foods i.e. breakfast cereals. A limitation of the study is that the participants were in good health and not obese. With increasing age and obesity there may be an increase in the rate of turnover of vitamin D resulting in lower serum concentrations (MacLaughlin and Holick 1985; Wortsman *et al.* 2000). There were some differences in BMI between groups at baseline owing to the small sample size. However adjusting for BMI in the analyses did not alter the conclusions, and in view of the findings in Chapter 3, the effects of BMI seem most pronounced in those with a BMI>30kg/m^2.

In conclusion, the present study shows that consumption of a malted milk drink fortified with a low dose of either D_2 or D_3 leads to significant improvements in serum 25-OH-D concentrations. It therefore can be concluded that dietary vitamin D_2 could be used at levels likely to be consumed in the diet to test whether supplementation

influences cardiovascular risk markers. The results indicate that a daily intake of 10 µg is more likely to be effective in improving vitamin D status than one of 5 µg.

Chapter 5

The effect of low dose vitamin D_2, provided in a fortified malted milk drink, on cardiovascular risk markers (DRISK study)

5.1 Introduction

Marked seasonal variations in serum 25-OH vitamin D (25-OH-D) concentrations occur which result in 55-70% and 17-39% of the UK population having values below 50 nmol/L and 25 nmol/L respectively in the winter months (Ruston 2004; Bates *et al.* 2014). Vitamin D insufficiency, as well as affecting bone health, may also increase risk of cardiovascular disease (CVD) (Wang *et al.* 2012a). Some evidence from long-term randomised controlled trials suggests that vitamin D supplementation (often in combination with calcium) reduces total mortality (Zheng *et al.* 2013; Bjelakovic *et al.* 2014; Chowdhury *et al.* 2014) but there is no clear evidence to support a beneficial effect on CVD incidence and mortality.

Vitamin D insufficiency might affect risk of CVD by altering vascular function and inflammation. There is some evidence from cross-sectional studies showing that low 25-OH-D concentrations are associated with higher BP (Judd *et al.* 2008; Scragg *et al.* 2007) endothelial dysfunction (Al Mheid *et al.* 2011; Jablonski *et al.* 2011), arterial stiffness (Al Mheid *et al.* 2011; Giallauria *et al.* 2012) and elevated levels of MMP-9 and C-reactive protein (Ngo *et al.* 2010; Timms *et al.* 2002). However, these associations may not be causal and could be confounded by associations with physical activity level (Al-Othman *et al.* 2012) and chronic disease. Some trials (Tarcin *et al.* 2009; Sugden *et al.* 2008; Harris *et al.* 2011) report improvements in vascular function and inflammation but others (Yiu *et al.* 2013; Witham *et al.* 2013a; Gepner *et al.* 2012) find no such effects. A few trials have produced un-interpretable results because they either have not controlled for sunlight exposure (Breslavsky *et al.* 2013; Gepner *et al.* 2012) or were poorly designed (lacking a placebo treatment) (Tarcin *et al.* 2009; Dong *et al*. 2010). Furthermore, most trials to date have used doses of vitamin D in the range of 63-125 µg/d (Gepner *et al.* 2012; Yiu *et al.* 2013), or a single dose of around 2500 – 7500 µg (Harris *et al.* 2011; Tarcin *et al.* 2009; Witham *et al.* 2010) and so are pharmacological rather than nutritional comparisons as the amounts are much greater that can be achieved through dietary intake. A need for a well-designed study in healthy older people is long overdue.

The aim of the study presented here was to ask the question “Does an intake of vitamin D that can be achieved through diet in the winter months improve endothelial

function and thus lower BP?". The decision to use ergocalciferol (D_2) was taken in order to exclude any possible effect of sunlight exposure; the dose level of 10 μg/d was selected on the basis of the trial described in Chapter 4. Flow mediated dilation of the brachial artery was chosen as the best measure of endothelial function as it is a well validated method of measuring the capacity of the vascular endothelium to produce the vasodilator nitric oxide (NO) (Green *et al.* 2014). BP is subject to substantial measurement error and observed bias. In order to overcome this, 24 h ABP monitoring was used. To exclude the known adverse effects of smoking on vascular function only non-smoking participants were invited. However, as age is a major predictor of future CVD, it was decided to recruit healthy men and women 50-70 y of age.

5.2 Methods

5.2.1 Hypothesis

Dietary vitamin D_2 provided in a malted milk drink in the winter months will lower BP and improve endothelial function in healthy older men and women.

Corollary: Dietary vitamin D_2 provided in a malted milk drink in the winter months will reduce chronic inflammation and slow arterial stiffening.

5.2.2 Objective

To conduct a randomised placebo controlled trial of an intake equivalent to 10 μg vitamin D_2/d, provided in a malted milk drink, in healthy men and post-menopausal women (50-70 y) during the winter months.

Primary outcome:

BP determined by 24 h ambulatory monitoring and endothelial function measured by FMD.

Secondary outcomes:

1) Indices of inflammation (hsCRP, MMP-9, and fibrinogen).
2) Lipid profile (TC, HDL-C and triglycerides) with TC:HDL-C as the major indicator of risk.
3) Arterial stiffness.

Exploratory outcomes

Plasma renin concentrations, factor VII coagulant activity ($FVII_c$), insulin sensitivity and β cell function assessed using the homeostatic model assessment (HOMA-2).

Markers of compliance to the intervention

The metabolites 25-OH-D_2, 25-OH-D_3, Ca^{2+}, and parathyroid hormone (PTH) were measured along with body weight. Any unused sachets of intervention product were returned and a tally of the number consumed recorded. Serum free vitamin D concentrations were estimated *post hoc* using published equations after determining VDBP and albumin concentrations.

5.2.3 Sample size

Sample size calculations were based on being able to detect a 1 unit SD change in FMD over the 12 wk intervention period. Assuming a mean FMD of 6.8% for healthy subjects, with an SD of 2.0, a sample size of 16 per group would be required to detect a 1.4% change in FMD with $\alpha = 0.05$ and 80% power, assuming two measurements are made on treatment. For 24 h ABP, a within-subject SD of 7 mm was estimated, and two measures at baseline and two on treatment would give power to detect a 5 mm change. Allowing for drop-outs, a target of recruiting 40 participants was set.

5.2.4 Ethical approval, clinical governance and R&D approval

Ethical approval was obtained from the London-Westminster Research Ethics Committee 1 (REC reference 11/LO/1626) on 06/12/2011 (**Appendix 19**). The trial was registered at http://www.controlled-trials.com and allocated a clinical trial registration number ISRCTN45316882 on 07/09/2012. Approval from the Guy's and St Thomas' Department of Research and Development (R&D) was obtained on 14/12/2011 (Trust R&D registration number RJ111/N332) (**Appendix 20**) and additional consent was given for analysis of VDBP. Participants gave written informed consent (**Appendix 21**) and received a small remuneration for taking part in the study.

5.2.5 Study design

A parallel design randomised placebo-controlled trial was selected as the seasonal nature of the study would make a cross-over design unsuitable. Participants were

randomly allocated to treatment using minimisation for gender and ethnicity on the MinimPy Program 0.3. The treatment allocation was done by a member of KCL staff who was unconnected with the study. The member of staff had the treatment allocation (A or B) for each of the subject codes, but was blinded to the identity of A or B in terms of whether it was active or placebo treatment. The allocation of treatment (A or B) was held by a code-breaker who was a member of GlaxoSmithKline staff unrelated to the study and was concealed until the study was completed and data for the primary and secondary outcomes had been entered into the statistical database (29/11/2013).

Measurements were made at baseline and after 6 and 12 wk of intervention; in the case of 24 h ABP a further baseline measurement was made following the screening visit (**Figure 5.1**). The study took place between 20/01/2012 and 03/05/2012, and between 04/01/2013 and 30/04/2013, both periods when UVB exposure was low in the UK, and this was verified by data from the Meteorological Office (**Appendix 12**).

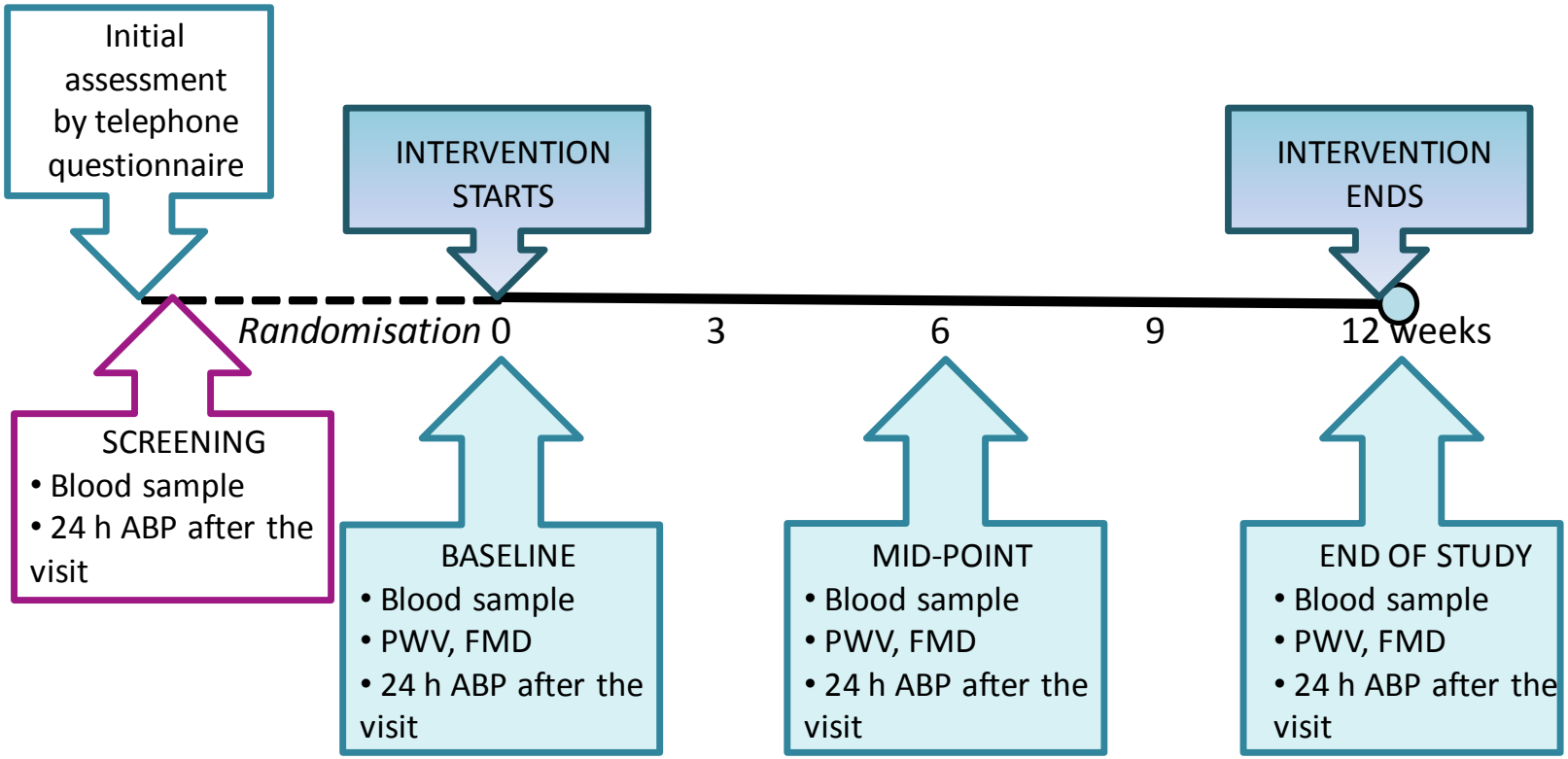


Figure 5.1 Study design.

Screening blood samples tested for glucose, lipids, liver function, 25-OH-D and haematology. ABP, Ambulatory blood pressure monitoring; FMD, flow-mediated dilation; PWV, Pulse Wave Velocity; Anthropometric measurements including height, weight, BMI and waist circumference taken at every visit, including screening; Blood metabolites at baseline, mid-point and end of study tested for vitamin D binding protein, 25-OH-D_2 and 25-OH-D_3, Ca^{2+}, PTH, glucose, C-peptide, high sensitivity C-reactive protein, MMP-9, lipids (total cholesterol, HDL cholesterol and triglycerides), renin, fibrinogen and factor VII coagulant activity. Lifestyle questionnaire returned and checked at screening and mid-point. Physical activity questionnaire completed at baseline and mid-point.

5.2.6 Test materials

A malted milk drink (Horlicks™, GlaxoSmithKline, Middlesex, UK) was used as a vehicle to supply the vitamin D, and the placebo treatment was an identical drink without added vitamin D. Participants were asked to consume three drinks/wk. The material to constitute the drink was provided in single serves in plain silver sachets: for the active treatment each sachet provided 24 μg vitamin D_2/serving. Participants were given a supply of 42 sachets at the start of the intervention and requested to return any unused sachets at the end of the study. They were provided with instructions to make the drinks up with hot water and not to add milk. Each 25 g sachet provided: 96 kcal, 2.4 g protein, 19.3 g carbohydrate (of which sugars, 8.8 g), 0.8 g fat (of which saturates, 0.3 g) and 0.1 mg sodium. The ingredients list was: wheat flour, malted barley, dried whey, calcium carbonate, sugar, vegetable fat, dried skimmed milk, salt, acidity regulator (potassium bicarbonate).

5.2.7 Recruitment of participants

The study aimed to recruit healthy men or post-menopausal women aged 50-70 y. Exclusion criteria were: a reported history of angina pectoris, MI, stroke, peripheral vascular disease, arterial fibrillation, congenital heart defects or congenital heart disease, ABP >150/95 mm Hg (assessed by ABP monitoring), current use of medication for lowering blood cholesterol (statins) or BP, type 1 or type 2 diabetes mellitus (fasting blood glucose >7.0 mmol/L), chronic renal, liver or inflammatory bowel disease, current cigarette smoker, underweight or morbidly obese (Body Mass Index <18.5 and >35 kg/m^2), an overall risk of CVD over the next 10 y of >20% assessed according to QRISK2 (ClinRisk Ltd 2013), prolonged exposure to high UVB light since Nov 2011 (or since Nov 2012 for the second cohort), going to a lower latitude country, or using a tanning sun-bed during the study period, serum 25-OH-D at screening >65nmol/L, intolerance to study product (lactose, milk protein), taking vitamin and mineral supplements (including cod-liver oil) or prescription calcium/vitamin D, unwillingness to restrict consumption of oily fish to no more than 2 portions per wk, consuming soya milk, unwillingness to follow the protocol and/or give informed consent.

Participants were recruited via an email sent to the staff and student population at KCL (**Appendix 22**), posters at KCL (**Appendix 23**), and a London Metro newspaper advertisement (**Appendix 24**). Subjects expressing interest were provided with a study information sheet (**Appendix 25**) and asked to complete a telephone/email questionnaire (participants had the option as to whether to complete the questionnaire sent by email or to ring up and complete it over the telephone) if still interested (**Appendix 26**). Those who appeared eligible and willing to participate were invited to attend a screening appointment either at the MRU in Franklin-Wilkins Building at KCL, or in the Clinical Research Facility (CRF) at St Thomas' Hospital to further assess their suitability for inclusion in the study. BP and anthropometric measurements were taken, along with a blood sample to ascertain that haematology, liver function, lipid profile, plasma glucose and 25-OH-D concentrations were within prescribed limits (**Appendix 16**). The subject was fitted with an ABP monitor at the end of the visit and asked to wear it for the next 25 hours. They were also asked to bring in a completed lifestyle questionnaire containing a FFQ (**Appendix 9**), which they had been sent prior to their appointment to determine their usual dietary intake of vitamin D and medical history. A flow chart detailing the process is shown in **Figure 5.2**.

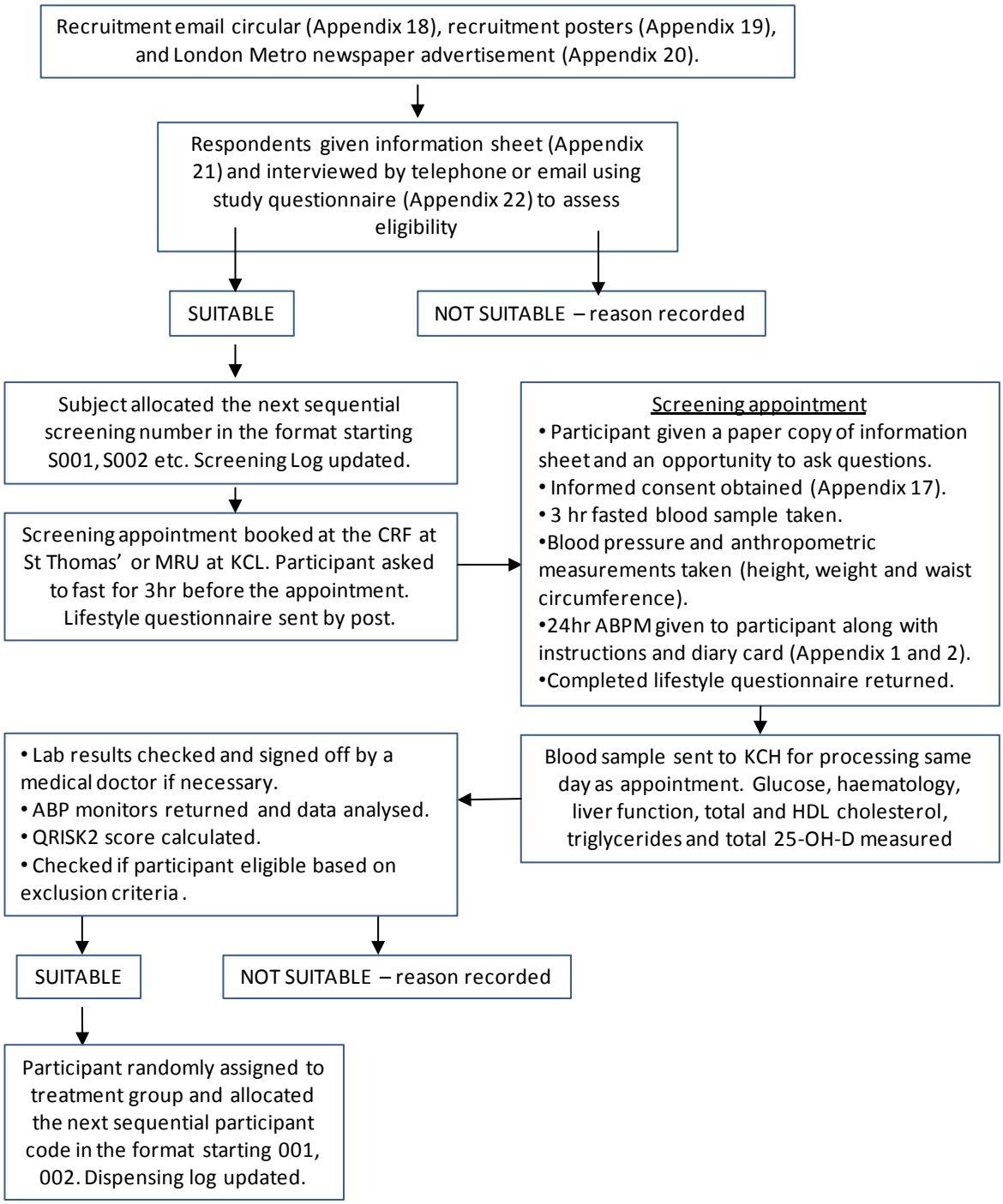


Figure 5.2 Flow diagram of participants through the study. KCL, King's College London; MRU, Metabolic Research Unit; ABP, Ambulatory blood pressure monitoring.

5.2.8 Dietary intakes and physical activity

Prior to the intervention (screening or baseline) and during the study (6 wk visit), participants completed a validated physical activity questionnaire (PAQ) (**Appendix 27**) (Craig *et al.* 2003) and a lifestyle questionnaire (**Appendix 9**) similar to that used in the

European Prospective Investigation in Cancer (EPIC) study (Bingham *et al.* 2001) which included an FFQ validated for vitamin D intake in the UK population (Crowe *et al.* 2011), and asked about their medical history, previous smoking habits and educational attainment. Section 4.2.7 described how the lifestyle questionnaire data were analysed and habitual dietary intake determined. Physical activity data were analysed using the International Physical Activity Questionnaire (IPAQ) scoring protocol to determine median metabolic equivalents (MET) – min/wk.

5.2.9 Ambulatory blood pressure monitoring

Measurements of 24 h ABP were made following screening, baseline, 6 wk and 12 wk visits using A&D TM-2430 (A & D Instruments, 24-26 Blacklands Way, Abingdon Business Park, Abingdon Oxfordshire, OX14 1DY) devices (graded AA by the British Hypertension Society) (Williams *et al.* 2004) in accordance with UK guidelines (O'Brien *et al.* 2005). Further details are provided in section 2.2.4. The ABP monitor was programmed to take measurements every 30 min during the day (0700-2200) and hourly at night (2200-0700). At the end of the screening visit, participants were instructed how to use the monitor and were fitted with it. They were given a brief instruction card (**Appendix 4**) and diary card (**Appendix 5**) with their monitor. In a small number of cases, it was inconvenient for the participant to wear the monitor immediately after the visit (but not after the screening visit). If this occurred, it was checked that the participant had a friend or relative who could fit the monitor for them when convenient with the help of the instruction card. The trained researcher also went over with the participant the important points to remember in fitting the monitor correctly, and demonstrated this at the end of the visit to remind them. During readings taken by the monitor the participant was instructed to stop what they were doing and relax their arm. They were asked to complete the diary card following each measurement to report what they were doing immediately prior to the measurement (lying down, sitting, standing or walking plus any other details or problems that may have occurred) and to record when they went to bed and woke up. After fitting the monitor, the researcher recorded details of the monitor number, where and when (date and time) the monitor could be collected and the contact information of the participant. A courier was booked and the monitor was returned directly to the researcher at KCL. Monitors were usually collected within a day or two

of the measurements being taken. The data were downloaded onto a computer, analysed using TM-2430-13 Doctor-Pro Software, and the first 3 readings obtained were discarded, leaving the remaining readings to be checked by the author. If it was considered that there were insufficient accurate measurements (at least 22 awake measurements and 6 asleep measurements), or the equipment had failed to work, the participant was requested to wear the monitor again for 24 h as soon as possible.

5.2.10 Vascular function and measurements made on blood samples

Before each clinic visit at baseline, 6 wk and 12 wk, participants were asked to avoid strenuous physical activity, foods high in fat, caffeine and alcohol on the day prior to the visit and to fast overnight from 22:00 drinking nothing but water until attending the CRF at St Thomas' Hopsital the following morning. Each visit began with a short questionnaire in the ward to check that the participant was not taking any nutritional supplements, and to ask whether they had started taking any medication or had experienced prolonged sun exposure since the last visit. Measurements of weight, waist circumference and seated BP were then taken and a blood sample collected using the vacutainer method (Becton-Dickinson, Oxford). For all but one participant, blood samples were collected in the seated position. Both serum (after being left to stand at room temperature for 30 min) and plasma were centrifuged for 15 min at 1300 g then separated from the gel section/haematocrit and stored pending analysis at -70°C. The following analytes were determined at baseline, 6 wk and 12 wk: 25-OH-D_2 and 25-OH-D_3 by uHPLC/MS/MS, VDBP, serum Ca^{2+}, PTH, C-peptide, glucose, hs-CRP, renin, fibrinogen, $FVII_c$, MMP-9, TC, HDL-C and triglycerides. **Table 5.1** shows the methods used and where the analyses were conducted, and gives reference to the appropriate section in Chapter 2 where the methods are described in detail. Care was taken to ensure that the PTH sample was stored at -70°C within two hours of collection. Details on the specific vacutainers used and blood handling methods including centrifuge speed and time and temperature the sample was spun at can be found in **Appendix 7**. Following the blood sample, participants were asked to rest in the supine position on a ward bed for at least 10 min and measurements of supine BP and arterial stiffness (see section 2.2.2) were made. The participant then walked to the ultrasound room and rested for a further 15 min prior to a measurement of flow mediated dilatation (FMD) of the brachial artery (section 2.2.1). Measurements of FMD

were usually made on the right arm and blood was generally taken from the left arm, ensuring that FMD measurements were not in the arm from which blood had been taken earlier. Following the measurements, participants were fitted with an ABP monitor to wear over the next 25 h (section 5.2.9). They were then offered a breakfast of tea/coffee, cereal and bread with butter and jam, marmite or peanut butter.

Table 5.1 Summary of the biochemical measurements and methods.

Analyte	Method	Section where details can be viewed	Where analysis was conducted
PTH	Immunoassay	2.4.7	KingsPath
Renin	Chemiluminescence immunoassay	2.4.8	KingsPath
Calcium	Colourimetric assay	2.4.5	KingsPath
hsCRP	Immunoturbidimetry	2.4.9	KingsPath
MMP-9	ELISA	2.4.10	KingsPath
C-peptide	Chemiluminescent immunometric	2.4.11	KingsPath
25-OH-D_2 and D_3	uHPLC-MS/MS	2.4.6.1	LGC
Total 25-OH-D	Chemiluminescent immunoassay	2.4.6.2	KingsPath
VDBP	ELISA	2.4.12	KingsPath
Fibrinogen	Clotting time assay	2.4.11	KCL
$FVII_c$	Clotting time assay	2.4.11.1	KCL
Glucose	Enzymatic assay	2.4.1	KingsPath
Cholesterol	Enzymatic assay	2.4.4.1	KingsPath
HDL cholesterol	Enzymatic assay	2.4.4.2	KingsPath
Triglycerides	Enzymatic assay	2.4.4.3	KingsPath

PTH, parathyroid hormone; hsCRP, high sensitivity C-reactive protein; MMP-9, matrix-metalloproteinase 9; VDBP, vitamin D binding protein; $FVII_c$, Factor VII coagulant activity; KingsPath, King's College Hospital; KCL, King's College London. LGC, LGC Testing (Formerly HFL Sports Science), Cambridgeshire. ELISA, enzyme-linked immunosorbent assay; uHPLC-MS/MS, ultra-high pressure liquid chromatography tandem mass spectrometry.

5.2.11 Calculation of free 25-OH-D

Free vitamin D was defined as circulating 25-OH-D not bound to albumin or VDBP. It was calculated using the method described by Powe *et al* (Powe *et al.* 2013) using known affinity-binding constants for albumin and VDBP: Directly measured and calculated free 25-OH-D are strongly correlated (Powe *et al.* 2013).

5.2.12 Statistical analyses

Statistical analysis of the data was conducted using SPSS for Windows Version 21.0. Standard distributional checks were made, and where appropriate, analyses were attempted following $\log_e$ transformation. Where values were below the limit of detection, the limit of detection was assigned. In order to test whether the two treatment groups differed at baseline, comparisons were made using the independent samples *t*-test, Mann-Whitney U test or a Fisher's exact test for categorical variables. The treatment effect was defined as the difference compared to placebo. It was decided in the final statistical analysis plan to focus on 24 h SBP and FMD as the two primary outcomes to avoid penalties associated with multiple comparisons for day-time and night-time values as well as DBP. Data were analysed on an intention to treat basis using the SPSS General Linear Model (GLM) with the mean treatment as the dependent variable, the treatment allocation as the predictor factor, age, gender and BMI as covariates, and the mean baseline value as an offset variable. A main effects model was used, and pairwise contrasts were performed between treatment groups. The SPSS syntax is shown below:

```
GENLIN MeanTreatment24hSBP BY TreatmentGrp (ORDER=ASCENDING) WITH Age
A_BMI Sex
  /MODEL TreatmentGrp Age A_BMI Sex INTERCEPT=YES OFFSET=meanblspbl
 DISTRIBUTION=NORMAL LINK=IDENTITY
  /CRITERIA SCALE=MLE COVB=MODEL PCONVERGE=1E-006(ABSOLUTE) SINGULAR=1E-
012 ANALYSISTYPE=3(WALD) CILEVEL=95 CITYPE=WALD LIKELIHOOD=FULL
  /EMMEANS TABLES=TreatmentGrp SCALE=ORIGINAL COMPARE=TreatmentGrp
CONTRAST=PAIRWISE PADJUST=LSD
  /MISSING CLASSMISSING=EXCLUDE
  /PRINT CPS DESCRIPTIVES MODELINFO FIT SUMMARY SOLUTION.
```

Where variables could not be normalised by log transformation, medians and inter-quartile ranges were determined and non-parametric tests performed, including the

Mann-Whitney U test to compare changes in the outcomes from baseline to post-intervention between groups.

5.3 Results

5.3.1 Participant details

There were 179 respondents to the advertisement, 133 completed the initial questionnaire, 53 attended a screening visit and 41 were randomly allocated to treatment. Two participants in the placebo group withdrew their consent from the study for personal reasons; one following a dog-bite and the other owing to an unrelated medical condition that required pain-killing medication. Data were available for analysis on the primary outcome for 18 and 21 participants in the placebo and vitamin D_2 groups. The CONSORT diagram is displayed in **Figure 5.3** and the details of the participants by randomised treatment are shown in **Table 5.2**. The mean age was 60 y, 46% were in paid employment and 32% were retired. Generally a high proportion had completed higher education. Although the mean BMI was in the normal range, the average waist circumference indicated a fairly high prevalence of central adiposity. The participants were predominantly normotensive, but had moderately elevated serum cholesterol (>5.0 mmol/L) and a total cholesterol:HDL cholesterol ratio >3.0. Their 10 y risks of CVD estimated by QRISK2 were typical of adults of their age in the UK, being higher in men (10%) than women (6.7%). At screening, the mean (SD) 25-OH-D concentration in nmol/L was 38 (14) and did not differ between groups.

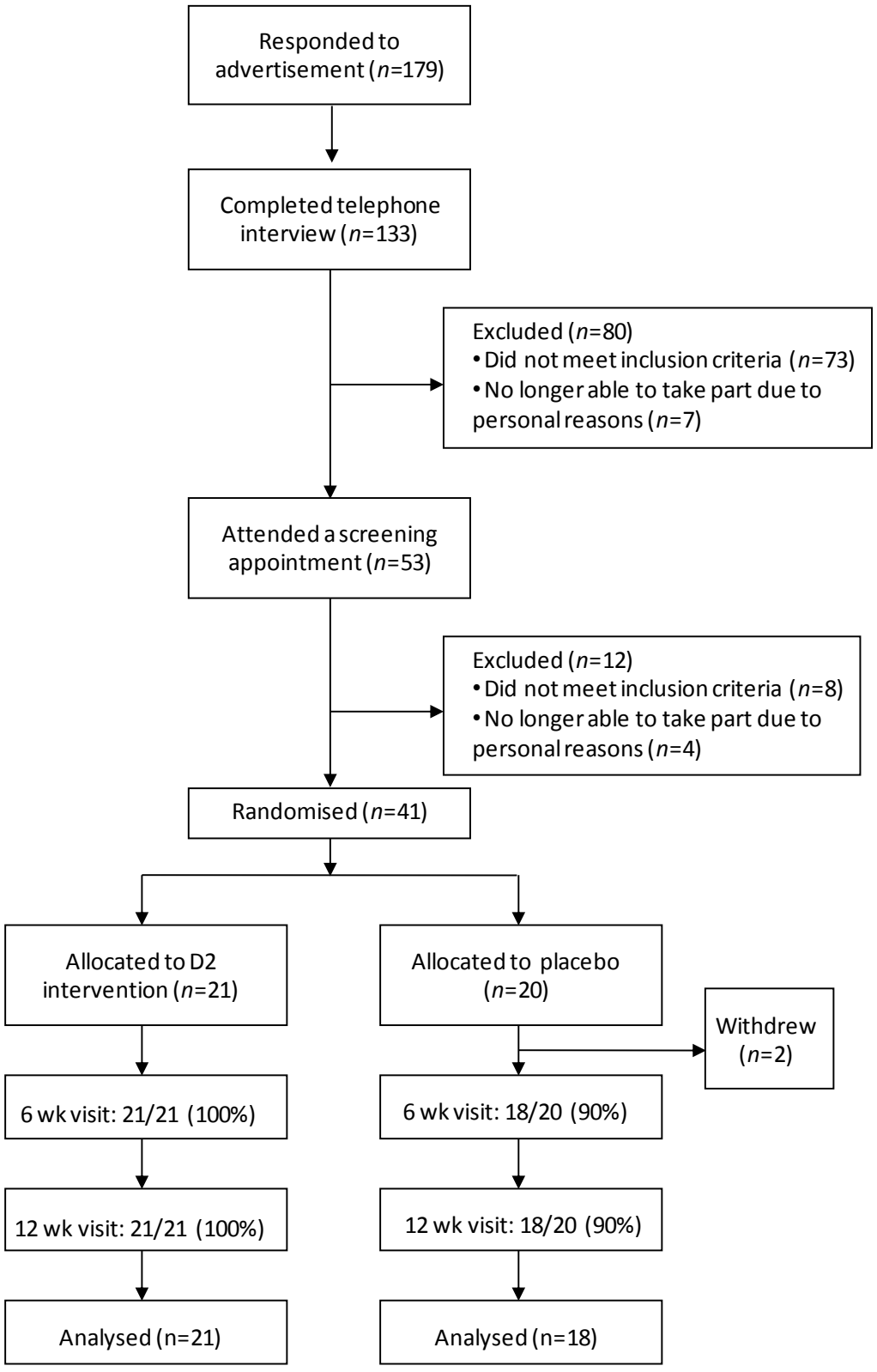


Figure 5.3 CONSORT flow diagram for the trial

Table 5.2 Details of the participants at randomisation.

	Men		Women	
	Placebo (*n*=10)	**Vitamin D (*n*=10)**	**Placebo (*n*=10)**	**Vitamin D (*n*=11)**
Age (y)	57 (6)	61 (4)	60 (5)	59 (6)
Ethnicity				
White	8 (80%)	9 (90%)	9 (90%)	11 (100%)
Black	0 (0%)	1 (10%)	0 (0%)	0 (0%)
Asian	2 (20%)	0 (0%)	0 (0%)	0 (0%)
Far East	0 (0%)	0 (0%)	1 (10%)	0 (0%)
Other	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Height (m)	1.77 (0.07)	1.77 (0.04)	1.64 (0.08)	1.63 (0.04)
Weight (kg)	76.8 (13.7)	78.7 (11.4)	62.4 (9.1)	65.1 (9.0)
BMI (kg/m^2)	24.3 (3.5)	25.0 (3.2)	23.3 (3.3)	24.5 (3.2)
Waist circumference (cm)	91.3 (12.1)	92.8 (11.2)	80.7 (6.8)	83.3 (11.3)
Seated SBP (mm Hg)	122 (9)	128 (11)	120 (17)	118 (13)
Seated DBP (mm Hg)	81 (8)	81 (9)	75 (12)	73 (7)
QRISK-2 (%)	9.6 (5.1)	10.9 (3.6)	7.4 (4.1)	5.9 (3.6)
Glucose (mmol/L)	4.9 (0.6)	5.2 (0.5)	5.0 (0.6)	5.4 (0.5)
Total cholesterol (mmol/L)	5.5 (1.0)	4.9 (1.0)	6.0 (1.0)	5.4 (0.7)
HDL-C (mmol/L)†	1.4 (0.4)	1.5 (0.3)	1.9 (0.5)	1.7 (0.5)
LDL-C (mmol/L)	3.4 (0.8)	3.0 (0.7)	3.5 (0.9)	3.3 (0.7)
TC:HDL-C ratio	3.88 (1.02)	3.35 (0.76)	3.43 (1.02)	3.36 (0.98)
Haemoglobin (g/dL)	14.2 (1.0)	14.7 (1.1)	13.4 (0.7)	13.4 (0.8)
WBC (giga/L)	5.24 (0.79)	5.52 (0.99)	5.99 (0.99)	5.71 (1.22)
RBC (tera/L)	4.74 (0.45)	4.96 (0.28)	4.43 (0.30)	4.61 (0.47)
Total protein (g/L)	71 (2)	71 (3)	70 (3)	69 (3)
Albumin (g/L)	44 (2)	44 (1)	44 (2)	43 (1)
Bilirubin (µmol/L)‡	11 (8-11)	12 (11-14)	9 (8-10)	8 (6-9)
ALP (IU/L)	65 (14)	57 (8)	68 (16)	73 (20)
AST (IU/L)	25 (7)	24 (6)	22 (3)	21 (5)
GGT (IU/L)†	20.3 (9.59)	20.20 (7.96)	18.0 (14.5)	13.14 (5.4)
Total 25-OH-D (nmol/L)	31.3 (14.4)	37.2 (12.4)	40.0 (12.1)	39.3 (9.3)
Education (highest achieved)				
GCE/O-level	0 (0%)	2 (20%)	2 (20%)	2 (18.2%)
A-level	1 (10%)	1 (10%)	1 (10%)	4 (36.4%)
HND, Diploma	3 (30%)	2 (20%)	1 (10%)	2 (18.2%)
Degree	5 (50%)	5 (50%)	4 (40%)	3 (27.3%)
None of these/other	1 (10%)	0 (0%)	2 (20%)	0 (0%)
Employment status				
In paid employment	5 (50%)	4 (40%)	6 (60%)	4 (36.4%)
Unemployed	1 (10%)	2 (20%)	0 (0%)	0 (0.0%)
Retired	3 (30%)	3 (30%)	3 (30%)	4 (36.4%)
Housewife/husband	0 (0%)	0 (0.0%)	1 (10%)	2 (18.2%)
Other	1 (10%)	1 (10%)	0 (0%)	1 (9.1%)

Number of subjects (%) or mean values (SD) or †geometric mean (approx SD) or ‡median (IQR). Comparisons between groups show no significant differences using independent samples *t*-test, Mann-Whitney *U* test, or Fisher's exact test as appropriate; QRISK-2, 10 year risk of cardiovascular disease. ALP, alkaline phosphatase; AST, aspartate transaminase; GGT, gamma glutamyl transferase.

5.3.2 Body weight, waist circumference, dietary intake of vitamin D and calcium, and physical activity during the study.

Table 5.3 details the body weight, waist circumference, vitamin D and calcium intake, and total physical activity of participants during the study, which did not change significantly. Participants were given 42 sachets of the intervention product and returned approximately six indicating that they had consumed 3 sachet/wk for 12 wk. Dietary intake of vitamin D excluding the intervention product was unchanged.

Table 5.3 Changes in body weight, waist circumference, dietary intake and physical activity

	Placebo (*n*=18)	Vitamin D_2 (*n*=21)	Treatment Effect (95% CI)	*P* value
Body weight (kg)				
Baseline	69.6 (13.3)	71.4 (11.8)		
Midpoint	70.5 (13.0)	71.5 (12.1)		
Endpoint	70.3 (13.0)	71.5 (12.5)		
Mean treatment	70.4 (13.0)	71.5 (12.3)	0.29 (-0.35, 0.93)	0.36
Waist circumference (cm)†				
Baseline	85.5 (9.9)	88.7 (12.5)		
Midpoint	84.6 (9.3)	87.8 (12.8)		
Endpoint	85.8 (9.7)	87.6 (12.5)		
Mean treatment	85.2 (9.3)	87.7 (12.5)	-0.1% (-1.8, 1.6)	0.93
Number of Sachets out of 42 returned	5.7 (2.0)	5.3 (1.9)	-0.3 (-1.6, 0.9)	0.60‡
Dietary vitamin D intake (µg/d)†				
Baseline	3.2 (2.2)	2.4 (1.7)		
Treatment	2.8 (1.2)	2.4 (1.3)	-4.0% (-21.3, 17.1)	0.68
Treatment + intervention	2.8 (1.2)	12.6 (1.3)		
Calcium intake (mg/d)				
Baseline	929 (281)	932 (272)		
Treatment	976 (289)	964 (332)	-21.5 (-195, 152)	0.85
Physical activity (MET – min/wk) ¶				
Baseline	261 (127, 440)	297 (146, 594)		
Treatment	419 (240, 494)	366 (185, 524)	-6.0% (-55.0, 42.9)	0.44

Values are mean (SD); †Geometric mean (approx SD) and treatment effect calculated as percentage using log transformed data. Treatment effects and probability from Generalised Linear Model with baseline value as offset adjusted for age and gender (physical activity also adjusted for BMI); ‡two sample *t*-test. ¶ median (IQR) and Mann Whitney test used to calculate probability; MET, Metabolic Equivalent of Task, the ratio of the physical activity metabolic rate to the resting metabolic rate (BMR).

5.3.3 Vitamin D status

The mean serum concentrations of 25-OH-D_2, 25-OH-D_3 and total 25-OH-D at baseline were 2.8, 39.8 and 42.6 nmol/L respectively, with 18% <25 nmol/L and 72% <50 nmol/L (**Table 5.4**). Serum vitamin D metabolite concentrations remained low and unchanged in the placebo group. In the D_2 group, the median 25-OH-D_2 metabolite concentrations increased from 2.4 (IQR 2.4, 2.4) to 25.9 (IQR 18.6, 33.4) nmol/L, appearing to plateau between 6 and 12 wk of supplementation (**Figure 5.4**). The change in total 25-OH-D in the D_2 group (16.2, 95% CI 10.4, 22.0) was significantly greater (*P*<0.001) than that in the placebo group (-3.3, 95% CI -9.6, 3.0). There was a non-significant trend for 25-OH-D_3 to fall in both groups compared with the baseline measurement. After 12 wk of D_2 supplementation, total 25-OH-D concentrations had increased from 40.8 nmol/L at baseline to 59.2 nmol/L such that none of the participants had 25-OH-D values <25 nmol/L, but 24% still remained <50 nmol/L. Individual results are presented in **Appendix 28**. There was a trend (*P*=0.104) for VDBP to fall in the D_2 group compared with placebo. Levels of free 25-OH-D rose significantly in the D_2 group compared with placebo (*P*<0.001). There were no significant treatment effects for serum Ca^{2+} or plasma PTH concentrations (**Table 5.5**). However, a Mann-Whitney U test on the change in PTH brought the *P* value for PTH close to significance (*P* = 0.065).

Table 5.4 Effects of an intake equivalent to 10 µg vitamin D_2/d *vs.* placebo on 25-OH-D metabolites, vitamin D binding protein and free 25-OH-D concentrations in healthy men and women aged 50-70 y.

	Placebo (*n*=18)	Vitamin D_2 (*n*=21)	Treatment effect (95% CI)	*P* value
25-OH-D_2 (nmol/L) †				
Baseline	2.4 (2.4, 3.0)	2.4 (2.4, 2.4)		
6 wk	2.4 (2.4, 3.2)	22.9 (16.3, 30.1)		
12 wk	2.7 (2.4, 5.0)	25.9 (18.6, 33.4)		
Mean on treatment	2.7 (2.4, 3.7)	22.3 (19.6, 30.9)		
Change	10.7% (-19.0, 40.4)	199% (169, 221)		
Adjusted change	15% (-13.0, 43.4)	195% (169, 221)	180% (141, 218)	1.22E-07
25-OH-D_3 (nmol/L)‡				
Baseline	35.2 (25.4)	35.08 (14.7)		
Midpoint	31.0 (24.8)	31.40 (10.4)		
Endpoint	33.1 (21.4)	32.59 (8.7)		
Mean on treatment	33.5 (24.1)	33.41 (12.0)		
Change	-5.1% (-10.3, 0.5)	-4.8% (-9.7, 0.4)		
Adjusted change	-5.4% (-10.4, -0.3)	-4.8% (-9.5, 0.1)	0.6% (-6.4, 7.6)	0.868
25-OH-D (nmol/L)				
Baseline	44.8 (26.2)	40.8 (14.4)		
Midpoint	40.2 (24.9)	55.2 (15.7)		
Endpoint	42.3 (21.2)	59.2 (12.6)		
Mean on treatment	41.2 (22.6)	57.2 (13.5)		
Change	-3.6 (-10.4, 3.2)	16.5 (10.2, 22.8)		
Adjusted change	-3.3 (-9.6, 3.0)	16.2 (10.4, 22.0)	19.5 (10.8, 28.2)	1.10E-05
VDBP (mg/L)				
Baseline	355.3 (157)	382.3 (188.9)		
Midpoint	380.12 (189.3)	366.0 (178.2)		
Endpoint	359.6 (191.3)	373.9 (199.9)		
Mean on treatment	369.9 (188.8)	370.0 (186.9)		
Change	14.6 (-9.3, 38.5)	-12.3 (-34.5, 9.8)		
Adjusted change	13.9 (-8.6, 36.4)	-11.8 (-32.6, 9.0)	-25.7 (-56.7, 5.3)	0.104
Free 25-OH-D (pmol/L)‡				
Baseline	6.23 (3.93)	5.98 (7.11)		
Midpoint	5.31 (4.12)	8.67 (7.38)		
Endpoint	6.23 (4.14)	9.49 (8.20)		
Mean on treatment	5.87 (3.91)	9.12 (7.74)		
Change	-6% (-21, 12)	52% (30, 78)		
Adjusted change	-6% (-22, 10)	42% (28, 56)	48% (27, 69)	1.09E-05

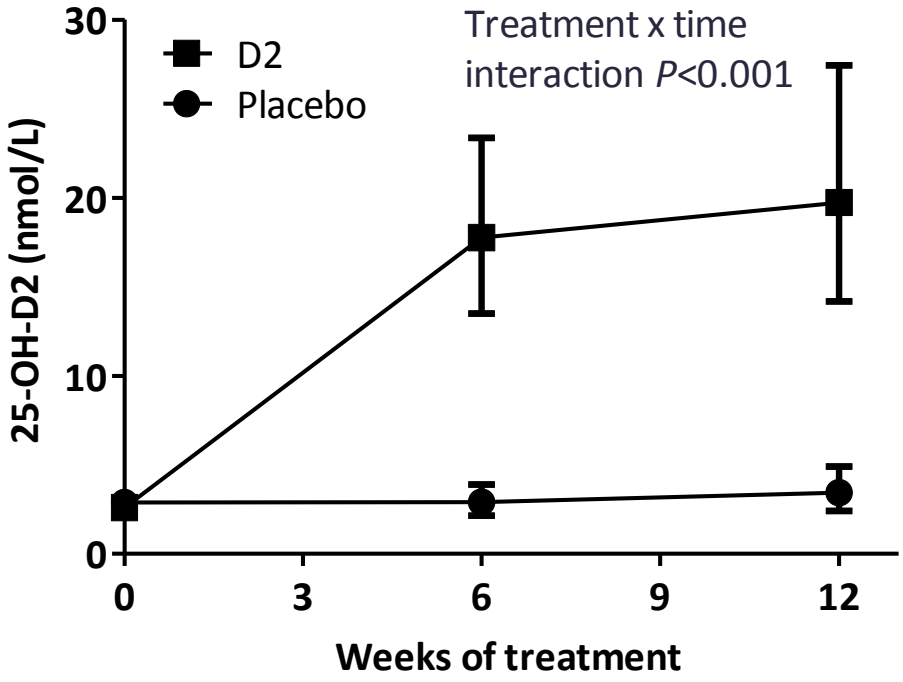
Values are mean (SD); Treatment effect and probability from Generalised Linear Model with baseline value as offset and age, BMI and gender as covariates, adjusted changes are the marginal means adjusted for covariates; †Median (IQR), treatment effect calculated as percentage using log transformed data and probability calculated using Mann-Whitney U test; ‡Geometric mean (approx SD) and treatment effect calculated as percentage using log transformed data. VDBP, vitamin D binding protein.

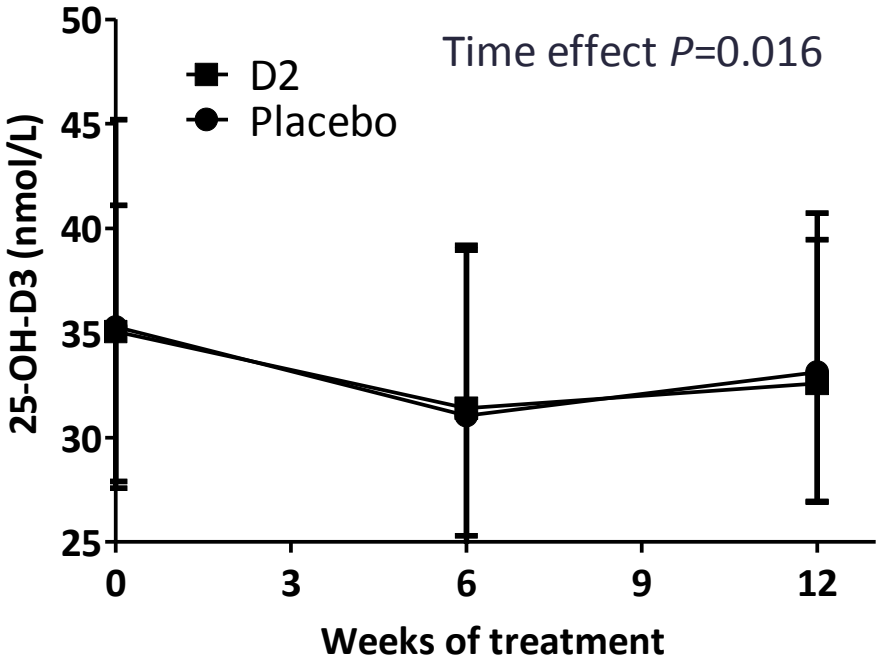
Table 5.5 Effects of an intake equivalent to 10 µg vitamin D_2/d *vs.* placebo on serum calcium and plasma parathyroid hormone (PTH) in healthy men and women aged 50-70 y.

	Placebo (*n*=18)	Vitamin D_2 (*n*=21)	Treatment effect (95% CI)	P value
Ca^{2+} (mmol/L)				
Baseline	2.26 (0.09)	2.23 (0.08)		
6 wk	2.25 (0.09)	2.22 (0.06)		
12 wk	2.24 (0.10)	2.24 (0.07)		
Treatment	2.25 (0.09)	2.23 (0.06)		
Change	-0.02 (-0.04, 0.01)	0.01 (-0.02, 0.03)		
Change adjusted	-0.00 (-0.04, 0.01)	0.00 (-0.02, 0.03)	0.02 (-0.02, 0.05)	0.381
PTH (ng/L)†				
Baseline	38.9 (18.2)	39.9 (15.6)		
6 wk	38.6 (23.4)	36.1 (16.6)		
12 wk	40.6 (25.2)	34.1 (19.3)		
Treatment	39.8 (23.5)	35.5 (16.4)		
Change (%)	2.1 (-12.4, 19.0)	-10.8 (-22.8, 2.8)		
Change adjusted (%)	2.6 (-12.1, 17.1)	-11.8 (-25.3, 1.7)	-14.3 (-34.4, 5.8)	0.163

Mean values (SD); †Geometric mean (approx SD) and treatment effect calculated as percentage using log transformed data. Treatment effect from Generalised Linear Model with baseline value as offset, covariates age, BMI and gender. Adjusted changes are the marginal means adjusted for covariates.

Figure 5.4 **Serum concentrations of 25-OH-D_2 (panel A) and 25-OH-D_3 (panel B) in healthy men and women not exposed to UVB following placebo (*n*=18) or an intake equivalent to 10 µg vitamin D_2/d (*n*=21) for 12 wk in the winter months. Values are geometric means with 95% CI. Treatment x time and time effects are from repeated measures ANOVA.**





5.3.4 Blood pressure, flow mediated dilation and arterial stiffness

Table 5.6 shows the changes in BP measured by 24 h ABP monitoring. Both SBP and DBP fell on D_2 treatment compared with placebo: mean differences (95% CI) in mm Hg, adjusted for age, gender and BMI were -4.3 (-7.3, -1.2; *P*=0.007) and -2.8 (-5.4, -0.2, *P*=0.032), respectively. There was no evidence to indicate that the lower BP was related to changes in heart rate. The mean differences in day-time and night-time SBP in mm Hg were -3.3 (95% CI -6.7, 0; *P*=0.05) and -4.7 (95% C -7.7, -1.7; *P*=0.002) respectively (**Figure 5.5**) (**Appendix 29**). **Table 5.7** shows the results for plasma renin, which remained unchanged during the study.

Table 5.6 Effects of an intake equivalent to 10 µg vitamin D_2/d *vs.* placebo on 24 h systolic and diastolic BP and heart rate in healthy men and women aged 50-70 y.

	Placebo (*n* = 18)	Vitamin D_2 (*n* = 21)	Treatment effect (95% CI)	*P* value
SBP (mm Hg)				
Baseline 1	119 (11)	120 (12)		
Baseline 2	118 (14)	119 (10)		
Baseline mean	119 (12)	120 (10)		
6 wk	121 (11)	118 (11)		
12 wk	122 (11)	118 (8)		
Treatment mean	121 (10)	118 (9)		
Change	2.39 (-0.05, 4.83)	-2.07 (-4.33, 0.19)	-4.5 (-7.6, -1.3)	0.005
Adjusted change	2.27 (0.04, 4.51)	-1.97 (-4.04, 0.09)	-4.3 (-7.3, -1.2)	0.007
DBP (mm Hg)				
Baseline 1	73 (7)	73 (8)		
Baseline 2	71 (7)	73 (7)		
Baseline mean	72 (6)	73 (7)		
6 wk	75 (7)	72 (8)		
12 wk	73 (8)	72 (7)		
Treatment mean	74 (7)	72 (7)		
Change	2.36 (0.18, 4.55)	-0.76 (-2.79, 1.26)	-3.1 (-5.9, -0.3)	0.029
Adjusted change	2.18 (0.33, 4.04)	-0.61 (-2.33, 1.11)	-2.8 (-5.4, -0.2)	0.032
24 h heart rate (bpm)				
Baseline 1	72 (6)	68 (8)		
Baseline 2	71 (8)	69 (8)		
Baseline mean	72 (7)	69 (7)		
6 wk	72 (6)	67 (8)		
12 wk	73 (6)	71 (7)		
Treatment mean	72 (6)	69 (7)		
Change	0.97 (-0.95, 2.89)	-0.21 (-1.99, 1.56)	-1.2 (-3.6, 1.3)	0.345
Adjusted change	0.85 (-0.89, 2.59)	-0.11 (-1.71, 1.50)	-1.0 (-3.4, 1.4)	0.433

Mean values (SD); Treatment effect from Generalised Linear Model with baseline value as offset. Adjusted changes are the marginal means adjusted for the covariates age, BMI and gender.

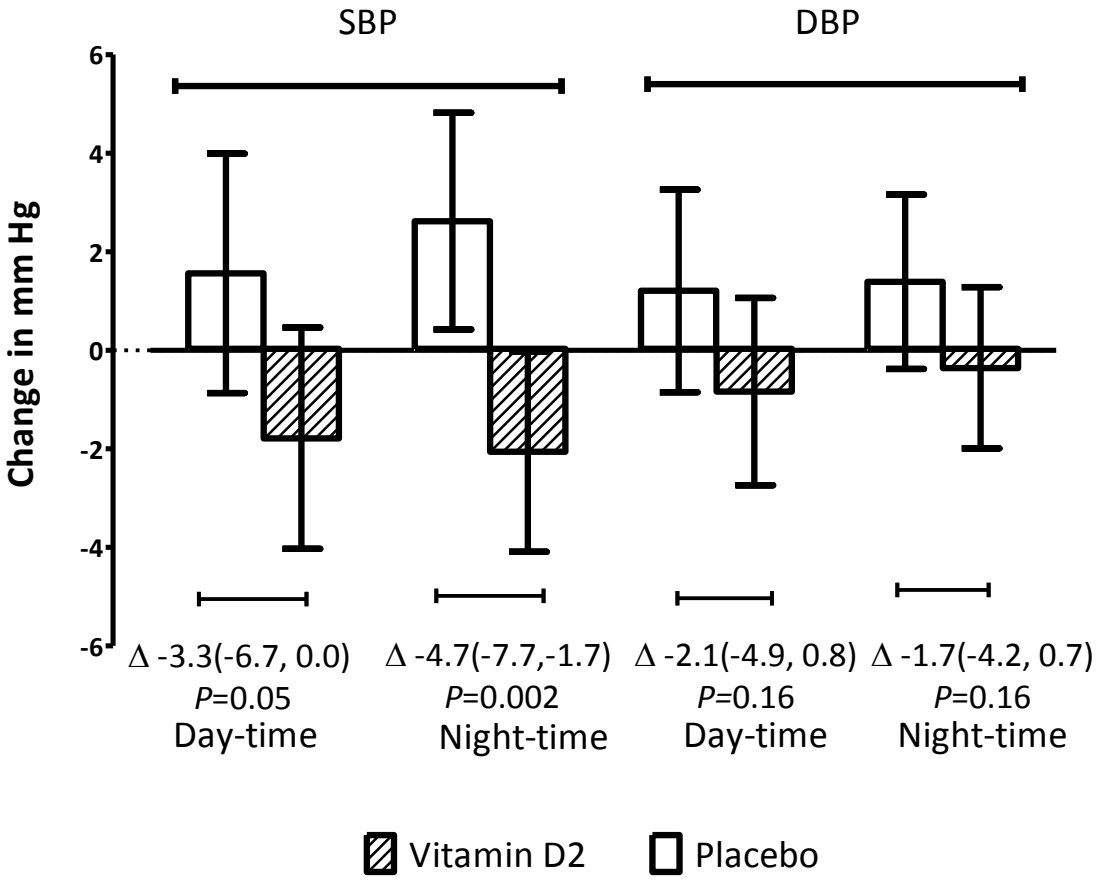


Figure 5.5 Mean changes (95% CI) in day-time and night-time systolic (SBP) and diastolic (DBP) BP in 21 and 18 participants allocated to an intake equivalent to 10 µg vitamin D_2/d or placebo respectively. Data were analysed using the SPSS Generalised Linear Model using the mean value on treatment regressed against treatment allocation, age, BMI, gender and mean baseline value as offset.

Table 5.7 Effects of an intake equivalent to 10 µg vitamin D_2/d *vs.* placebo on plasma renin concentrations in healthy men and women aged 50-70 y.

	Placebo (*n* = 18)	Vitamin D_2 (*n* = 21)	Treatment effect	*P* value
Renin (MIU/L)				
Baseline	13.5 (8.5, 18.2)	16.4 (9.9, 20.5)		
6 wk	12.1 (7.7, 17.6)	11.2 (7.4, 16.9)		
12 wk	11.1 (8.9, 19.4)	10.6 (8.6, 19.4)		
Treatment mean	12.8 (7.7, 18.5)	10.7 (8.8, 16.4)	-9.8% (-39.5, 19.9)	0.360

Median values (IQR). Treatment effect (95% CI) from the Generalised Linear Model using the logged mean value on treatment regressed against treatment allocation, age, BMI and gender with mean baseline value as offset. *P* value calculated using Mann-Whitney U test.

Table 5.8 shows the changes in FMD (%) and PWV (m/sec) over the 12 wk study period. The mean value among participants (3.8%) was lower than reported in previous studies from our laboratory where values are typically in the range of 6%, indicating some impairment of endothelial function among the participants. A total of 56% of participants had an FMD below 4% at baseline. There were no significant changes over the treatment period and the average FMD at each time point differed by less than 1% FMD unit between groups. The mean treatment effect on FMD with 95% CI was -0.17% (-1.62, 1.28; *P*=0.815). PWV remained unchanged in the placebo group but fell slightly in the vitamin D_2 group by -0.30 m/sec (95 CI -0.60, -0.002). However, the difference between treatments of -0.30 m/sec (-0.74, 0.14; *P*=0.184) did not achieve statistical significance.

Table 5.8 Effects of an intake equivalent to 10 µg/d vitamin D_2 *vs*. placebo on brachial artery diameter (BAD), flow mediated dilation (FMD) and carotid to femoral pulse wave velocity (PWVc-f)

	Placebo (*n*=18)	Vitamin D_2 (*n*=21)	Treatment effect (95% CI)	*P* value
BAD (mm)				
Baseline	3.60 (0.75)	3.56 (0.72)		
Midpoint	3.50 (0.76)	3.71 (0.66)		
Endpoint	3.52 (0.75)	3.69 (0.73)		
Mean on treatment	3.51 (0.74)	3.70 (0.66)		
Change	-0.08 (-0.29, 0.13)	0.14 (-0.06, 0.33)	0.23 (-0.06, 0.51)	0.118
Adjusted change	-0.10 (-0.30, 0.11)	0.15 (-0.04, 0.34)	0.24 (-0.04, 0.52)	0.094
FMD (%)				
Baseline	4.00 (2.51)	3.72 (2.63)		
Midpoint	5.56 (2.82)	4.46 (3.28)		
Endpoint	4.30 (2.53)	4.12 (2.19)		
Mean treatment	4.93 (2.10)	4.29 (2.33)		
Change	0.93 (-0.30, 2.16)	0.57 (-0.57, 1.71)	-0.36 (-1.94, 1.21)	0.650
Adjusted change	0.83 (-0.22, 1.88)	0.66 (-0.32, 1.63)	-0.17 (-1.62, 1.28)	0.815
$PWV_{c\text{-}f}$ (m/sec)				
Baseline	7.79 (1.15)	7.55 (0.98)		
Midpoint	7.81 (1.32)	7.30 (1.14)		
Endpoint	7.71 (1.30)	7.24 (1.14)		
Mean treatment	7.76 (1.28)	7.27 (1.12)		
Change	-0.03 (-0.37, 0.32)	-0.28 (-0.59, 0.04)	-0.25 (-0.69, 0.19)	0.264
Adjusted change	0.00 (-0.32, 0.32)	-0.30 (-0.60, -0.002)	-0.30 (-0.74, 0.14)	0.184

Mean values (SD). Treatment effect (95% CI) from Generalised Linear Model with baseline value as offset. Adjusted changes are the marginal means adjusted for the covariates age, BMI and gender.

5.3.5 Haemostatic and inflammatory risk factors and serum lipids

Table 5.9 shows the changes in haemostatic and inflammatory variables over the 12 wk study period. There were no significant treatment effects on hsCRP, fibrinogen or $FVII_c$. MMP-9 fell by 19.6% (-37.8, -1.4, *P*=0.035) in the D_2 group compared to placebo. **Table 5.10** presents the results for fasting lipids. HDL-C, LDL-C and TC increased in the D_2 group compared to placebo; differences in marginal means (95% CI) in mmol/L or as a percentage, were 6.9% (95% CI 0.8, 12.9; *P*=0.026), 0.2 (0.0, 0.5; *P*=0.038) and 0.3 (95% CI -0.0, 0.6; *P*=0.035) respectively. However, the TC:HDL-C ratio did not differ (*P*=0.581).

Table 5.9 Effects of an intake equivalent to 10 µg vitamin D_2/d *vs.* placebo on fibrinogen, factor VII coagulant activity ($FVII_c$), high sensitivity C-reactive protein (hsCRP) and matrix metalloproteinase-9 (MMP-9).

	Baseline	6 wk	12 wk	Treatment mean	Treatment effect	*P* value
Fibrinogen (g/L) ‡						
Placebo *n*=18	3.59 (3.28-4.08)	4.01 (3.44-4.40)	3.68 (3.44-3.93)	3.85 (3.56-4.20)		
Vitamin D_2 *n*=21	3.76 (3.33-3.98)	3.57 (3.24-3.91)	3.62 (3.32-4.01)	3.63 (3.33-3.78)	-7.2% (-18.2, 3.8)	0.185
$FVII_c$ (%)						
Placebo *n*=18	126 (31)	133 (29)	129 (31)	131 (29)		
Vitamin D_2 *n*=21	143 (37)	143 (49)	144 (50)	143 (45)	-5.0 (-20.7, 10.6)	0.528
HsCRP (mg/L) ‡						
Placebo *n*=14	0.70 (0.50-1.20)	0.90 (0.38-1.40)	0.63 (0.30-1.20)	0.65 (0.35-1.35)		
Vitamin D_2 *n*=19	0.75 (0.32-1.45)	0.70 (0.30-1.54)	0.76 (0.32-1.35)	0.84 (0.29-1.30)	-2.8% (-32.4, 26.9)	0.827
MMP-9 (µg/L)†						
Placebo *n*=18	216 (89)	238 (127)	223 (132)	233 (117)		
Vitamin D_2 *n*=21	264 (84)	235 (108)	226 (88)	233 (90)	-19.6% (-37.8, -1.4)	0.035

Mean values (SD); †Geometric mean (approximate SD) and treatment effect calculated as percentage using log transformed data; ‡ median (IQR) and treatment effect calculated as percentage using log transformed data. Treatment effects were derived from SPSS Generalised Linear Model regressed against age, BMI, gender with baseline value as offset. For hsCRP high value outliers were excluded.

Table 5.10 Effects of an intake equivalent to 10 µg vitamin D_2/d *vs.* placebo on serum lipids

	Baseline	6 wk	12 wk	Treatment mean	Treatment effect (95% CI)	*P* value
TC: HDL-C ratio						
Placebo *n*=18	3.5 (0.7)	3.6 (0.8)	3.5 (0.8)	3.51 (0.79)		
Vitamin D_2 *n*=21	3.2 (0.6)	3.2 (0.7)	3.2 (0.6)	3.21 (0.63)	-0.0 (-0.2, 0.1)	0.581
Total cholesterol (mmol/L)						
Placebo *n*=18	5.4 (0.9)	5.2 (0.9)	5.4 (1.0)	5.32 (0.89)		
Vitamin D_2 *n*=21	4.9 (0.6)	5.0 (0.7)	5.2 (0.7)	5.15 (0.71)	0.3 (-0.02, 0.6)	0.035
HDL-C (mmol/L)†						
Placebo *n*=18	1.6 (0.4)	1.5 (0.4)	1.6 (0.4)	1.54 (0.38)		
Vitamin D_2 *n*=21	1.6 (0.3)	1.6 (0.4)	1.7 (0.3)	1.63 (0.35)	6.9% (0.8, 12.9)	0.026
LDL-C (mmol/L)						
Placebo *n*=18	3.3 (0.7)	3.1 (0.7)	3.2 (0.7)	3.16 (0.72)		
Vitamin D_2 *n*=21	2.9 (0.5)	3.0 (0.6)	3.2 (0.7)	3.07 (0.63)	0.2 (0.0, 0.5)	0.038
Triglycerides (mmol/L)†						
Placebo *n*=18	1.1 (0.6)	1.1 (0.6)	1.2 (0.6)	1.15 (0.58)		
Vitamin D_2 *n*=21	0.9 (0.3)	0.9 (0.3)	0.8 (0.2)	0.89 (0.26)	-2.7% (-16.1, 10.7)	0.689

Values are mean (SD); †Geometric mean (approximate SD) with treatment effect calculated as percentage from log transformed data; Treatment effects (95% CI) derived from Generalised Linear Model regressed against age, BMI, gender with baseline value as offset.

5.3.6 Fasting glucose and indices of insulin secretion and sensitivity

Table 5.11 shows the changes in plasma glucose, C-peptide (as a marker of insulin secretion), β cell function and insulin sensitivity. The participants were normoglycaemic and estimates of insulin secretion and sensitivity were normal. There were no significant differences between treatment groups.

Table 5.11 Changes in plasma glucose, C-peptide and estimates of β cell function and insulin sensitivity following an intake equivalent to 10 µg/d vitamin D_2 *vs.* placebo for 12 wk.

	Placebo (*n* = 18)	Vitamin D_2 (*n* = 21)	Treatment effect	P value
Glucose (mmol/L)				
Baseline	4.8 (0.5)	5.3 (0.6)		
6 wk	4.9 (0.4)	5.2 (0.6)		
12 wk	4.8 (0.5)	5.2 (0.6)		
Mean on treatment	4.81 (0.40)	5.21 (0.52)	-0.01 (-0.20, 0.19)	0.932
C-Peptide† (nmol/L)				
Baseline	0.47 (0.20)	0.55 (0.19)		
6 wk	0.47 (0.24)	0.52 (0.18)		
12 wk	0.49 (0.26)	0.53 (0.22)		
Mean on treatment	0.48 (0.24)	0.53 (0.19)	-6.4% (-16.6, 3.8)	0.386
β cell function (%)†				
Baseline	105 (26)	99 (26)		
6 wk	104 (33)	99 (32)		
12 wk	113 (26)	97 (27)		
Mean on treatment	109 (27)	99 (27)	-4.2% (-12.1, 3.6)	0.287
Insulin sensitivity (%S)				
Baseline	106 (38)	87 (28)		
6 wk	108 (46)	90 (24)		
12 wk	102 (40)	89 (27)		
Mean on treatment	105 (42)	90 (23)	3.4 (-5.9, 12.6)	0.473

Values mean (SD); †Geometric mean (approx SD) and treatment effect calculated as percentage using log transformed data; Treatment effect from Generalised Linear Model with covariates age, BMI, gender and baseline value as offset.

5.4 Discussion and conclusion

This study adds to the current evidence that vitamin D insufficiency is prevalent during the winter in healthy British men and women in the age range 50-70 y. It shows that moderate supplementary intakes (10 μg/d) of vitamin D_2 from a fortified drink improve vitamin D status. The proportion of participants with plasma 25-OH-D <50 nmol/L fell from 71% to 24% with the vitamin D_2 intervention. However, free 25-OH-D may be a better indicator of physiological bioactivity in extra-renal tissues compared with total 25-OH-D (Glendenning *et al.* 2013). In young adults, free 25-OH-D concentrations are more strongly correlated with bone mineral density than total 25-OH-D concentrations (Powe *et al.* 2011). It is notable, therefore, that free 25-OH-D increased by 48.0% (95% CI 26.6, 69.3) with D_2 administration *vs*. placebo. The increment in free 25-OH-D is comparable to that reported in a study in which hip fracture patients were supplemented with either D_3 or D_2 at 25 μg/d for 3 months; the increases were 46% and 36% after D_3 and D_2 respectively (Glendenning *et al.* 2013). The findings of the present study indicate that an additional 10 μg/d was sufficient to improve vitamin D status both in terms of total 25-OH-D but also the proportion in the free fraction.

The salient finding was a -4.3 mm Hg treatment effect in 24 h SBP determined by ambulatory monitoring. Two primary outcomes (FMD and 24 h SBP) were specified and the probability was *P*=0.007 which is less than the critical value of *P*=0.025. However, the change in BP was slightly less than the 5 mm Hg for which the study was powered; the observed power for the change was 78%. Therefore a type 1 error may have occurred and the findings may be due to chance. BP increased in the placebo group whereas it fell in the vitamin D_2 group. It is well documented that BP increases between winter and spring (Modesti *et al.* 2013) but the reasons have been hitherto unexplained. The results presented here indicate that this may be due to vitamin D status. Whilst a few studies report that vitamin D supplementation lowers BP (Judd *et al.* 2010; Forman *et al.* 2013; Sugden *et al.* 2008), meta-analyses (Pittas *et al.* 2010; Kunutsor *et al.* 2014; Elamin *et al.* 2011; Witham *et al.* 2009; Wu *et al.* 2010) are equivocal. Most meta-analyses have concluded that there is no significant effect of vitamin D on BP (Pittas *et al.* 2010; Elamin *et al.* 2011; Kunutsor *et al.* 2014), but one found a significant decrease in DBP (-3.1 mm Hg, 95% CIs –5.5 to -0.6) (Witham *et al.* 2009) and another observed a decrease in SBP of 2.44 mm Hg (Weighted mean

difference -2.44, 95% CI -4.86, -0.02) (Wu *et al.* 2010). However, the meta-analysis by Witham *et al*. included one study which used UV radiation rather than giving a dose of vitamin D. Furthermore, the majority of studies included in all of the meta-analyses have measured clinic BP rather than using repeat 24 h ABP monitoring which is more reliable and less subject to bias (Mancia *et al.* 2007; Staessen *et al.* 1999). As a 20 mm Hg difference in usual SBP is associated with a doubling of risk CVD mortality (Lewington *et al.* 2002), the 4 mm Hg difference in SBP found in the present study may reduce risk of CVD mortality by 20%.

This change in SBP was not accompanied by changes in FMD which suggests that the BP lowering effect does not involve a change in the capacity of the vascular endothelium to synthesise nitric oxide. This lack of effect on FMD is in agreement with 3 recent studies showing no significant change in FMD after 62.5 µg vitamin D_3 daily for 4 months (Gepner *et al.* 2012), 125 µg vitamin D_3/d for 12 wk (Yiu *et al.* 2013), or a single oral dose of D_3 at 2500 µg or 5000 µg (Witham *et al.* 2010). However, FMD was more variable than anticipated and this may have reduced the power to detect a change. A few studies have claimed a positive effect of supplementation on FMD (Harris *et al.* 2011; Witham *et al.* 2012; Sugden *et al.* 2008; Tarcin *et al.* 2009), but all used very high doses of vitamin D; 1500 µg monthly D_3 (Harris *et al.* 2011), a single oral dose of 2500 µg D_2 (Witham *et al.* 2012; Sugden *et al.* 2008) or 7500 µg intra-muscularly monthly (Tarcin *et al.* 2009). Furthermore, three of these studies were conducted in groups with compromised health including stroke patients (Witham *et al.* 2012), overweight adults (Harris *et al.* 2011) and adults with type 2 diabetes (Sugden *et al.* 2008).

It would appear unlikely that the BP lowering effect can be explained by a change in renin secretion (Li 2003) as plasma renin concentrations remained unchanged in agreement with a report by Sugden *et al.* (Sugden *et al.* 2008). The BP lowering effect could be mediated by PTH. Associations between PTH and BP (Jorde *et al.* 2000; He *et al.* 2011) have been reported, and experimental studies have shown that PTH infusion leads to an increase in BP (Hulter *et al.* 1986). However, although there was a trend for PTH to fall on the active treatment, an effect mediated by PTH seems unlikely. The

BP lowering effect of vitamin D may be via direct modulatory effects on vascular smooth muscle cells or other pathways to yet be defined.

Few RCTs have measured arterial stiffness in response to vitamin D supplementation, and in agreement with the present study, most showed no change (Gepner *et al.* 2012; Larsen *et al.* 2012; Stricker *et al.* 2012; Yiu *et al.* 2013). In contrast, a trial in healthy black youth (Dong *et al.* 2010) did show an improvement in carotid-femoral PWV, measured using applanation tonometry and SphygmoCor software, from 5.41 ± 0.73 m/sec at baseline to 5.33 ± 0.79 m/sec post-test after 50 µg/d D_3 for 16 wk (P = 0.031). However, the size effect was small and this result could have arisen by play of chance. Furthermore, the study was only investigator-blinded, and was not placebo controlled (Dong *et al.* 2010). In the present study there was a trend for PWV to fall in the vitamin D_2 group (although not significantly different from placebo) which would be consistent with a fall in BP improving PWV (Nye 1964). PWV changes slowly over many years rather than over months, and a longer period of intervention may be required to show significant effects.

Some reports show that vitamin D supplementation decreases hsCRP (Timms *et al.* 2002; Asemi *et al.* 2013b; Witham *et al.* 2013b), but most find no effect (Belenchia *et al.* 2013; Jorde *et al.* 2010b; Muldowney *et al.* 2012; Pittas *et al.* 2007a; von Hurst *et al.* 2010; Wood *et al.* 2012; Yiu *et al.* 2013; Zittermann *et al.* 2009), as in the present study. Fibrinogen is also an acute phase protein and an independent risk factor for CVD that promotes thrombosis (Meade 1997). There is sparse data on fibrinogen's relationship with vitamin D. Cross-sectional studies show mixed results (Parikh *et al.* 2012; Shea *et al.* 2008) but a clinical trial in which 218 long-term hospital inpatients aged over 65 y were randomised to receive a similar dosage of 10 µg/d D_3, 30 µg/d D_3, or placebo for 6 months also showed no significant changes in fibrinogen (Bjorkman *et al.* 2009). Cross-sectional studies have found 25-OH-D to be inversely and independently associated with MMP-9 (Baker *et al.* 2014; Timms *et al.* 2002; Wasse *et al.* 2011). Expression of MMPs in atherosclerotic plaques by inflammatory cells may enhance the matrix breakdown in fibrous caps leading to an increased likelihood of cap rupture (Agewall 2006). In the present study, MMP-9 fell in the D_2 group compared to placebo (-19.6%, 95% CI -37.8, -1.4, P = 0.035). Although this was a secondary outcome

and there were multiple primary outcomes which increases the probability of this occurring at *P*=0.05 by chance, the finding is in agreement with a study by Timms *et al*. that reported reductions in MMP-9 of -57.84% and -64.68% when participants were supplemented for 1 y with three monthly injections of 1250 µg D_3 or 12.5 µg D_3, respectively (Timms *et al.* 2002). Another trial comparing 25 µg D_3 *vs*. placebo for 12 wk in participants with type 2 diabetes reported a significant difference in the change in MMP-9 on treatment (-2.3 ± 3.7) *vs*. control (0.44 ± 7.1), *P*=0.02 (Shab-Bidar *et al.* 2011). In contrast, Muldowney *et al*. failed to show a significant change in MMP-9 after 5, 10, or 15 µg/d of vitamin D_3 for 22 wk in 394 subjects (Muldowney *et al.* 2012). It is unknown as to what the mechanisms for a potential association between vitamin D and MMP-9 may be, although *in vitro* studies have indicated that vitamin D in its active form $1,25(OH)_2D$ down-regulates and suppresses MMP-9 production (Bahar-Shany *et al.* 2010; Anand and Selvaraj 2009; Coussens *et al.* 2009).

There were significant increases in TC, LDL-C and HDL-C in the D_2 group compared to placebo; mean differences between treatments were 5.6%, 6.3% and 6.9% respectively. However, there was no effect on the TC:HDL-C which is regarded as the most robust lipid metric of risk (Lewington *et al.* 2007), and lipid profile was a secondary outcome therefore significance may have occurred by chance. A meta-analysis of 10 RCTs suggested that vitamin D supplementation increased LDL-C by 0.084 mmol/L (95% CI, 0.014, 0.15), but there were no effects on other lipids (Wang *et al.* 2012a). In contrast, analysis of data from 600 postmenopausal women in the Women's Health Initiative (WHI) RCT which gave 1000 mg calcium and 10 µg D_3/d found a 0.12 mmol/L decrease in LDL-C (95% CI, 0.011, 0.22) compared with placebo (*P* = 0.03) (Schnatz *et al.* 2014).

No effect of D_2 supplementation on insulin sensitivity or β cell function was observed, consistent with a meta-analysis of 15 vitamin D supplementation RCTs (George *et al.* 2012).

Strengths and weakness.

The strength of the present study is that it was conducted double-blind in an older age group during the winter months and it used vitamin D_2 which meant that the findings

were not confounded by changes in vitamin D status due to sunlight exposure. Additional strengths were the use of repeated ABP monitoring and demonstration of compliance to the intervention using uHPLC tandem mass-spectroscopy. The short-term nature of the study is, however, a limitation. Furthermore, the participants who were predominantly white only showed mild vitamin D insufficiency compared to people with melanised skin and this limits the extent to which the findings can be generalised to the wider population. More marked effects may be found in individuals exhibiting a greater vitamin D insufficiency.

In conclusion, this study shows that an additional intake of 10 µg/d vitamin D_2 lowers SBP but does not influence FMD in predominantly normotensive healthy older men and women.

Chapter 6

The effect of low dose vitamin D_2, provided in a fortified malted milk drink, on mood and cognitive function (DRISK study)

6.1 Introduction

It has been observed that low serum 25-hydroxyvitamin D (25-OH-D) concentrations are associated with impairment of cognitive function (van der Schaft *et al.* 2013; Annweiler *et al.* 2013; Balion *et al.* 2012). However, many studies have not adjusted for important confounders such as gender, age, BMI, seasonality and education (van der Schaft *et al.* 2013). Furthermore, causality cannot be proven, and it may be that adults with cognitive decline have a lower vitamin D status owing to pre-existing disorders or less time exposed to sunlight. Despite this, there are reasons to believe that vitamin D may have a role in the brain; the enzyme 1α-hydroxylase which converts vitamin D to the active form, $1,25(OH)_2D$, and the VDR for $1,25(OH)_2D$ are widely distributed in the human brain in neurons and glial cells (Eyles *et al.* 2005). Furthermore, $1,25(OH)_2D$ has been found to have a number of roles in the brain including modulating calcium binding proteins which have neuroprotective roles (Alexianu *et al.* 1998), modulating the production of nerve growth factor (Neveu *et al.* 1994) and nitric oxide synthase (Garcion *et al.* 1998), and stimulating neurogenesis and some of its target gene products (McCann *et al.* 2008; Grant 2009).

The few vitamin D supplementation trials that have been conducted with cognitive function as an outcome have shown mixed results and were either not randomised or placebo-controlled (Annweiler *et al.* 2012; Przybelski *et al.* 2008) or had short follow-up periods of 6 wk or less which is unlikely to be long enough to see an effect (Dean *et al.* 2011; Przybelski *et al.* 2008). Trials assessing mood have also shown inconsistent results (Lansdowne and Provost 1998; Harris and Dawson-Hughes 1993; Jorde *et al.* 2008). Consequently, there is a need for a longer term, well-conducted, randomised, placebo-controlled trial to examine the effect of vitamin D supplementation in adults with lower 25-OH-D concentrations at baseline on mood and cognition (Annweiler *et al.* 2013). It was decided therefore to measure cognitive function in the participants who were enrolled in the vitamin D_2 supplementation trial described in Chapter 5.

6.2 Hypothesis

Vitamin D_2, provided in a malted milk drink in the winter months, will improve cognitive function in healthy, older men and women.

6.3 Objective

To measure cognitive function in participants enrolled into a randomised placebo controlled trial of an intake equivalent to 10 µg vitamin D_2/d, provided in a malted milk drink, in healthy men and post-menopausal women (50-70 y) during the winter months.

6.4 Methods

This study was conducted as part of the study described in Chapter 5. See section 5.2 for details of ethical approval, clinical governance and R & D approval, sample size, inclusion and exclusion criteria, recruitment and screening methodology, sample analysis and composition of malted milk drink.

6.4.1 Study design

The parts of the study that are relevant to this chapter are shown in **Figure 6.1**. Subjects were asked to complete a computerised cognitive function test (about 25 min) at the baseline and final 12 wk visit (section 6.4.2).

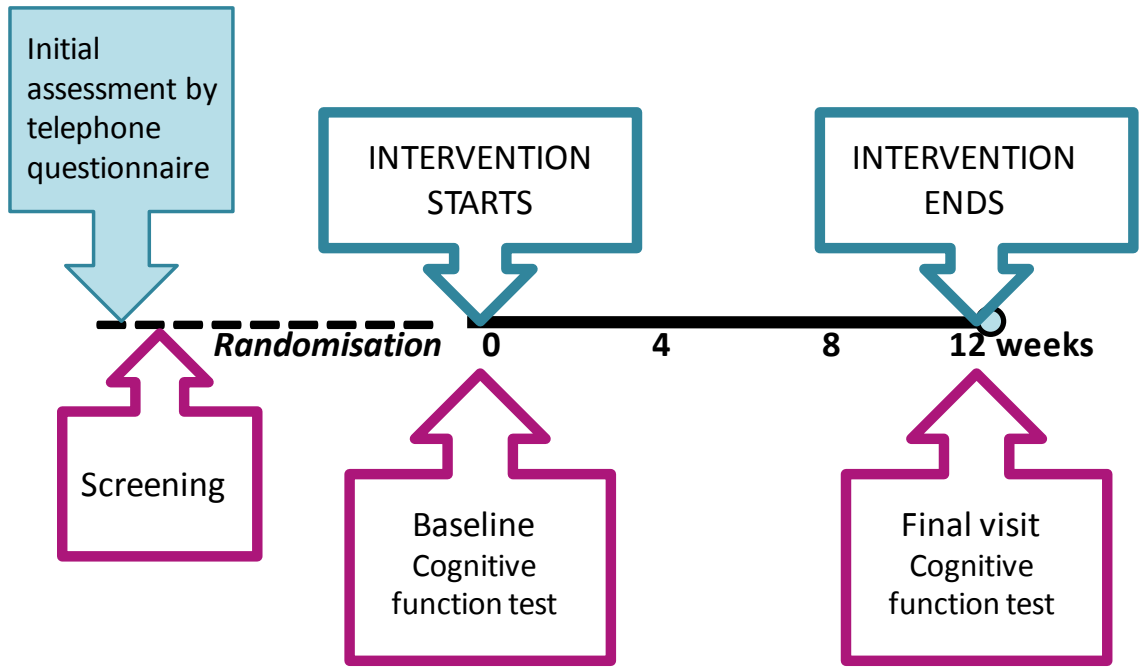


Figure 6.1 Outline of study design

6.4.2 Cognitive function test

Cognitive function was assessed with the Computerised Mental Performance Assessment System (COMPASS) (Northumbria University) which has previously shown sensitivity to interventions of other nutrients (Kennedy *et al.* 2010). The battery of tests consisted of a series of tasks to assess mood and cognition (including episodic memory, working memory and attention) which are described in sections 6.4.2.1 to 6.2.2.2, and took approximately 25 min to administer. The test was conducted under controlled conditions. Before the baseline and 12 wk clinic visits, participants were asked to avoid strenuous physical activity, foods high in fat, caffeine and alcohol on the day prior to the visit and to fast overnight from 22:00 drinking nothing but water until after their appointment. Tests always took place between 0900 and 1200. Participants were taken to a temperature controlled room, free from noise and disturbance, and left alone to complete the cognitive function test undisturbed. All tests were completed on the same laptop computer with the same mouse and Cedrus RB-530 response pad (this had buttons representing yes/no, left/right, blue/green/yellow/red and a central reaction time button) (**Figure 6.2**). Prior to the assessment, participants were instructed on how to use the response pad and were given a short 5 min training session on how to complete the telephone number, stroop, and peg and ball tasks.

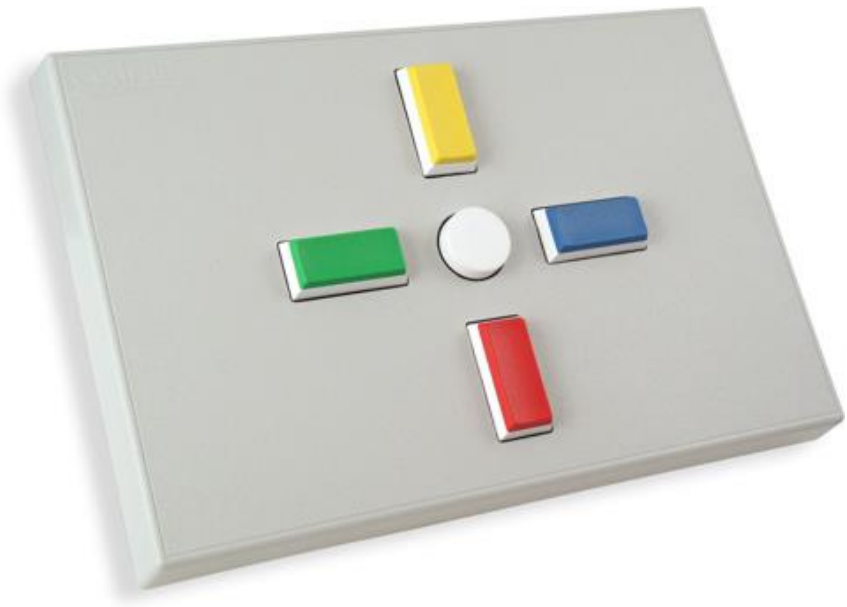


Figure 6.2 Cedrus RB-530 response pad which participants used in addition to the mouse for completing the cognitive function test.

6.4.2.1 Assessing mood

6.4.2.1.1 Bond Lader

Displayed on the screen was a line representing a scale, which had an adjective describing two generally opposing moods on each side. Using a mouse, participants were asked to click at a point on the scale which represented how they were feeling at that time. Each scale was scored out of 100 and in total there were 16 scales. Scores were split into those describing feelings of 'Alert', 'Calm' and 'Content', added together into 3 composite scores.

6.4.2.2 Assessing cognition

6.4.2.2.1 Simple reaction time (SRT)

This test consisted of an upwards pointing arrow appearing on the computer screen at irregular intervals. Participants were asked to respond by pressing the central white button on the response pad as quickly as they could once the arrow was displayed. The outcome of this task was the average reaction time in msec.

6.4.2.2.2 Telephone number working memory

A nine-digit telephone number was displayed on the screen for 5 seconds which the participant was required to memorise. After a further 5 seconds during which the screen was blank, a number pad appeared on the screen and participants had to enter as much of the number as they could remember using the mouse to select numbers on screen. Numbers could not be deleted or re-entered. The outcome for this task was the % accuracy overall and the overall reaction time in msec.

6.4.2.2.3 Stroop

This task involved a series of colour names (RED, YELLOW, GREEN, BLUE) being displayed on the screen. Each name was written in a red, yellow, green or blue coloured font. Participants were required to press on the response pad the button which corresponded with the colour of the font, not the colour the word depicted. Both congruent and incongruent stimuli were displayed. The outcome for this task was the % accuracy overall and the overall reaction time in msec.

6.4.2.2.4 Corsi blocks

The computer displayed 9 blue squares on a black background for this task. In a sequence, some of the blue squares changed to red and back to blue. The participants were required to remember this sequence and once it had finished to click on the blue squares that had changed colour, in the exact sequence that they had changed. The task started with only 4 blocks changing colour, and at each level was repeated 5 times. A block was added for each level after this until the participant was unable to remember 3 or more sequences at one level accurately. The score for this task was calculated as the average of the last 3 correctly completed trials. For example, if the participant correctly responded to all 5 level 4 trials and only 1 level 5 trial, their span score would be 4.3 [(4+4+5)]/3].

6.4.2.2.5 Peg and ball

This test consisted of two configurations displayed on the screen which each showed a series of 3 pegs containing 3 coloured balls (red, blue, green). The goal configuration was the one displayed at the top of the screen and the participant was required to arrange the balls on the bottom configuration to match with the position of those on

the goal configuration. They were asked to do this in as few moves as possible. Average thinking and completion times were calculated from 18 peg and ball presentations, discounting the first 2 practice trials. Total errors were calculated as the total number of moves in excess of the total number required to complete all 18 trials i.e. total number of moves in excess of 72.

6.4.2.2.6 Word presentation

Near the beginning of the cognitive function test a list of words was displayed on the screen, one word at a time. A total of 15 words were displayed with a 1 sec presentation time and a 1 sec inter-stimulus time.

6.4.2.2.7 Word recognition

This task involved a display on the screen, one at a time, of all target words that were shown during the word presentation in addition to an equal number of decoy words. Participants were required to indicate whether they had seen the word earlier or not by pressing on the middle button of the response pad. The outcome for this task was the % accuracy overall and the overall reaction time in msec.

6.4.3 Statistical analysis

Statistical analysis of the data was conducted using SPSS for Windows Version 21.0 on all participants for whom baseline and 12 wk data were available. Standard distributional checks were made, and where appropriate, analyses were attempted following log transformation. In order to test whether the two treatment groups differed in their cognitive function test scores at baseline, comparisons were made using the independent samples *t*-test, Mann-Whitney U test or a Fisher's exact test for categorical variables. Spearman's rank correlation was used to determine associations between baseline serum 25-OH-D and the cognitive function outcomes. Changes in each outcome from baseline to 12 wk in the D_2 group compared with placebo were assessed using a univariate analysis of covariance on the final scores adjusted for baseline scores, age, gender, BMI and education.

6.5 Results

A total of 41 participants were recruited and there were 2 drop-outs after the baseline visit (section 5.3.1). For one female participant, the laptop screen froze at baseline and the problem could not be fixed at the time. Therefore, the final analysis was conducted in 38 participants (*n*=21 in the D_2 group and *n*=17 in the placebo group) for whom data was available at baseline and 12 wk. There were 6 occasions over the 2 time points where the word recognition task did not display and therefore data is missing. Baseline characteristics are summarised in section 5.3.1 and **Table 5.2**. There were no significant correlations between baseline serum 25-OH-D concentrations and the mood or cognitive function outcomes. There were no differences in test scores at baseline between the D_2 and placebo groups. At baseline, males scored more highly than females on the Corsi Block Span test [mean difference (95% CIs), 1.5 (0.2, 2.9), *P*=0.026]. There were no other significant differences in scores between males and females. When age was split into two groups, one 50 to 60 years and the other 60 to 70 y, the younger group had a significantly higher Corsi Block Span score at baseline (*P*=0.010) and a significantly lower telephone overall reaction time score (*P*=0.032).

6.5.1 Effects of D_2 supplementation on mood

There were no significant treatment effects on mood assessed using the Bond Lader test (**Table 6.1**).

Table 6.1 Effects of an intake equivalent to 10 µg/d vitamin D_2 *vs*. placebo on mood

	Placebo	Vitamin D_2	Treatment effect (95% CI)	*P* value
Bond lader 'Alert' (*n*=38)				
Baseline	60.3 (15.7)	63.9 (17.7)		
Endpoint	61.6 (13.6)	61.7 (19.8)	-1.9 (-11.5, 7.6)	0.686
Bond lader 'Content' (*n*=38)				
Baseline	66.5 (14.5)	72.0 (17.8)		
Endpoint	69.0 (14.1)	71.6 (16.3)	-2.9 (-11.8, 6.0)	0.505
Bond lader 'Calm' (*n*=38)				
Baseline	63.0 (17.7)	63.8 (19.5)		
Endpoint	67.6 (17.6)	64.3 (20.7)	-5.9 (-18.1, 6.2)	0.325

Values mean (SD). Between group comparison by univariate ANCOVA adjusted for baseline value, age, gender, BMI and education.

6.5.2 Effects of D_2 supplementation on cognition

Table 6.2 Effects of an intake equivalent to 10 µg/d vitamin D_2 *vs*. placebo on cognition

	Placebo	Vitamin D_2	Treatment effect (95% CI)	*P* value
SRT Overall RT† (*n*=38) (msec)				
Baseline	323 (273, 398)	282 (271, 373)		
Endpoint	287 (252, 371)	300 (262, 330)	-1.9% (24.9, 28.1)	0.736
Tel % Correct (*n*=38)				
Baseline	36.8 (30.8)	26.7 (22.3)		
Endpoint	44.5 (34.5)	29.5 (29.3)	-5.7 (-20.5, 9.0)	0.434
Tel Overall RT‡ (*n*=38) (msec)				
Baseline	9414 (5487)	11159 (4955)		
Endpoint	8434 (1628)	10615 (7179)	13.9% (-8.2, 41.2)	0.227
Stroop % Correct† (*n*=38)				
Baseline	99.2 (98.3, 100)	99.2 (98, 100)		
Endpoint	99.2 (98.8, 100)	99.6 (98, 100)	2.7% (-10.2, 17.4)	0.831
Stroop Overall RT ‡ (*n*=38) (msec)				
Baseline	944 (165)	1012 (282)		
Endpoint	846 (116)	973 (339)	7.9% (-5.9, 23.6)	0.265
Corsi Block Span Score† (*n*=38)				
Baseline	5.7 (4.7, 6.0)	5.3 (2.7, 5.7)		
Endpoint	5.7 (5.5, 6.3)	5.0 (4.3, 5.7)	-9.2% (-27.3, 13.2)	0.976
P&B Average RT Thinking‡ (*n*=38) (msec)				
Baseline	5378 (2368)	5378 (2695)		
Endpoint	3944 (2419)	4024 (1502)	7.0% (-13.6, 32.6)	0.525
P&B Average RT Complete‡ (*n*=38) (msec)				
Baseline	18958 (6548)	15678 (8300)		
Endpoint	13630 (3636)	11968 (4183)	7.2% (-6.1%, 22.5%)	0.293
P&B Total Errors† (*n*=38)				
Baseline	2 (1,4)	7 (3,10)		
Endpoint	3 (1,7)	5 (2,11)	-36.6% (-75.8%, 65.8%)	0.638

	Placebo	Vitamin D_2	Treatment effect (95% CI)	*P* value
Word recognition % Correct‡ (*n*=32)				
Baseline	79.8 (10.2)	79.8 (13.0)		
Endpoint	82.3 (9.7)	80.6 (10.8)	-1.2% (-8.1%, 6.2%)	0.732
Word recognition Overall RT‡ (*n*=32) (msec)				
Baseline	1202 (338)	1327 (436)		
Endpoint	1133 (435)	1317 (411)	6.6% (-14.0%, 32.1%)	0.544

Values mean (SD); †Median (IQR), treatment effect calculated as percentage using log transformed data and probability calculated using Mann-Whitney U test; ‡Geometric mean (approx SD) and treatment effect calculated as percentage using log transformed data; Between group comparison by univariate ANCOVA adjusted for baseline, age, gender, BMI and education. SRT, simple reaction time; RT, reaction time; Tel, telephone; P&B, Peg and Ball.

There were no significant treatment effects on any of the cognitive function outcomes (**Table 6.2**). The Peg & Ball scores for average reaction time thinking and average reaction time complete significantly improved at 12 wk compare with baseline (*P* = 0.005 and *P* = 0.0003, respectively). There were no other significant differences between baseline and follow-up scores.

6.6 Discussion and conclusion

This study could find no effect of vitamin D_2 supplementation equivalent to 10 µg/d on measures of mood or cognition in healthy older adults in the winter months. Compared to norms and minimal performance levels (**Appendix 30**) from the Brain Performance and Nutrition Research Centre (BPNRC) where the COMPASS battery of tests was created, participants in this study scored close to or just below the norms for accuracy (% correct) in tests, but their reaction times were often below the minimal performance levels. For example, for the word recognition test, average overall reaction time scores at baseline and 12 wk were 1255 and 1229, respectively and the minimal performance level is 1104. This may be due to some of the participants being unfamiliar with using computers. For most tests, participants achieved higher scores on follow up compared to baseline as was expected due to an increased familiarity with the test, although this difference was only significant for the peg and ball task reaction times (Hausknecht *et al.* 2007). The peg and ball task is more complex than

some of the other tasks, and it required quite a lot of mouse use which some participants were not familiar with.

The findings of this study are in agreement with those from two other vitamin D supplementation trials (Dean *et al.* 2011; Przybelski *et al.* 2008) and a vitamin D and calcium trial (Rossom *et al.* 2012) which found no effect on cognitive function and are summarised in **Table 6.3**. The vitamin D and calcium RCT was conducted as part of the Women's Health Initiative in the US. A total of 2034 women were randomised to treatment (100 mg calcium carbonate and 10 µg vitamin D_3), and 2109 to placebo. Following enrolment onto the study between 1994 and 1999, participants completed annual cognitive assessments up until 2007. Global and domain-specific cognitive function were assessed using the Modified Mini-Mental State Examination (2MSE) and WHI Study of Cognitive Aging (WHISCA) cognitive battery, respectively (Rossom *et al.* 2012), but no significant changes occurred in the treatment group compared with placebo. There is evidence though that higher calcium concentrations are associated with poorer global cognitive function and cognitive decline (Schram *et al.* 2007) and therefore it is possible that any positive effect of vitamin D was masked by the negative effect of calcium. However, Dean *et al.* conducted a RCT giving only vitamin D_3 in capsules at an intake of 125 µg/d for 6 wk to 127 healthy younger adults with a mean age of 21.8 y. Participants were asked to complete a number of tests designed to assess working memory, response inhibition and cognitive flexibility, but no changes were found in any of the outcomes in the vitamin D group compared with placebo (Dean *et al.* 2011). Similarly, Przybelski *et al.* showed no effect of 1250 µg D_2 taken 3 times/wk for 4 wk on cognition in nursing home residents, although this study had no placebo group and so the results are questionable (Przybelski *et al.* 2008). A trial by Annweiler *et al.* did find improvements in cognitive performance, particularly in executive function, in older outpatients visiting a memory clinic who took 20 µg/d or 2500 µg/month of oral vitamin D_3 for 16 months. It is possible that longer term supplementation is needed to see an effect, although this trial was not randomised or placebo controlled and so further work is needed to explore this (Annweiler *et al.* 2012).

The finding of no effect of vitamin D supplementation on mood in the present study is consistent with the findings of other trials showing no effect on ratings of depression, anxiety or anger in younger adults (Dean *et al.* 2011), mental health scores of psychological well-being in elderly women (Dumville *et al.* 2006), or mood scores in 250 women aged 43 to 72 y who were in good general health (Harris and Dawson-Hughes 1993). In contrast, studies have shown a positive effect of 10 µg and 20 µg vitamin D_3 on mood in 44 healthy adults compared to placebo (Lansdowne and Provost 1998) and of 15 µg/d and 100 µg/d on wellbeing scores in endocrine outpatients (Vieth *et al.* 2004b). However, one of these trials did not have a placebo group (Vieth *et al.* 2004b) and both did not adjust for potential confounders (Vieth *et al.* 2004b; Lansdowne and Provost 1998).

Strengths and weakness.

Strengths of the present study are that it was a well-conducted placebo-controlled RCT with good compliance to the intervention, and it was conducted in a healthy, older age group with low baseline 25-OH-D levels during the winter months. Also, the results were adjusted for age, gender, BMI and education to rule out a confounding effect of these variables. The main weakness of the study is that it was not powered to detect a change in mood or cognitive function. It is possible that a larger sample size and longer intervention length may be needed to see an effect. In addition, the participants were all healthy with no known cognitive impairments and therefore the results may not be generalisable to other groups in the population.

In conclusion, this study has shown that an increased intake of vitamin D_2 of 10 µg/d on top of usual dietary vitamin D intake (3-4 µg/d) does not influence cognitive function or mood in healthy older adults.

Table 6.3 Summary of clinical trials investigating the effect vitamin D on cognitive function, including the trial described in this chapter.

Study	Population Characteristics	Treatment groups	No. of participants completed	Mean age y	Duration of study	Baseline and follow-up 25-OH-D (nmol/L)†		Relevant outcomes	Effect of treatment *vs* control
						Treatment	Control		
Current study (Unpublished data)	Healthy adults	Placebo or an intake equivalent to 10 µg/d D_2	39	59	12 wk	41 to 59	44 to 42	Mood and cognition	None
(Dean *et al.* 2011)	Healthy young adults	Placebo or 125 µg/d D_3	127	22	6 wk	76 to 98	77 to 75	Working memory, cognitive flexibility, response inhibition, depression, anxiety and anger	None
(Przybelski *et al.* 2008)	Nursing home residents	Control group with serum 25-OH-D >62 nmol/L took nothing (*n*=38) and another group (*n*=25) with serum 25-OH-D <62 nmol/L took 1250 µg D_2 3x/wk	61	87	4 wk	43 to 159	86 to 89	Cognition and behavioural disturbances	None
(Rossom *et al.* 2012)	Adult women	1000 mg calcium carbonate with 10 µg D_3/d or placebo	4143	71	Mean follow-up of 7.8 y	50 to ?	48 to ?	Cognitive impairment and cognitive function	None
(Annweiler *et al.* 2012)	Elderly outpatients	A D_3 group receiving 20 µg/d or 2500 µg/mo, and a control group receiving no vitamin D	44	81	16 mo	42 to 75¶	63 to 48 ¶	Cognitive and executive function	Significant improvement in cognitive performance and executive function

†Mean; ¶ Median

Chapter 7

Discussion

This thesis set out to investigate the effects of low intakes of vitamin D, as may be obtained in the diet, on vascular function and markers of inflammation. In order to overcome confounding by UVB exposure, the experimental studies were conducted in the winter months and vitamin D was provided as vitamin D_2. The ability of D_2 to raise serum 25-OH-D concentrations as effectively as D_3 has been questioned by Houghton and Vieth (Houghton and Vieth 2006) based on studies using pharmacological doses of the two forms, and a meta-analysis of RCTs (Tripkovic *et al.* 2012) concluded that D_3 was more efficacious. However, in the meta-analysis, the comparison between D_2 and D_3 for daily supplementation as opposed to bolus administration did not achieve statistical significance, and some studies included (Heaney *et al*. 2011, Romagnoli *et al*. 2008, Trang *et al*. 1998) used assays such as immunoassays that are unable to measure 25-OH-D_2 and 25-OH-D_3 separately, and may underestimate 25-OH-D_2 (Carter *et al.* 2007). The work described in this thesis used a 'gold-standard' uHPLC-MS/MS method with high precision and accuracy. The first RCT conducted gave 5 or 10 μg/d of D_2 or D_3 for 4 wk and increments from baseline compared with placebo following 5 and 10 μg/d of D_2 were (mean ± SEM) 9.4 ± 2.5 and 17.8 ± 2.4 nmol/L for 25-OH-D_2 and following 5 and 10 μg/d of D_3 were 15.1 ± 4.7 and 22.9 ± 4.6 nmol/L for 25-OH-D_3, respectively. The increment in 25-OH-D_2 following the dose equivalent to 10 μg/d *vs*. placebo in the second trial was 22.8 ± 2.0 nmol/L which is consistent with the findings of the first study and reaffirms the reliability of the results. The findings of the first study are displayed in **Figure 7.1** with previous placebo-controlled trials that have compared the ability of vitamin D_2 and D_3 to increase serum 25-OH-D concentrations at doses in the nutritional range (5-25 μg/d) in healthy adults (Biancuzzo *et al.* 2010; Holick *et al.* 2008). A study by Glendenning *et al*. which reports changes in 25-OH-D_2 and 25-OH-D_3 following 25 μg/d is excluded from the figure because it was in a group of very old women (aged 83 ± 8 y) who had sustained a hip fracture and it was uncontrolled having no placebo group (Glendenning *et al.* 2013). Logan *et al.* found 25 μg/d D_3 taken for 25 wk to be more effective than 25 μg/d D_2 at maintaining autumn and winter serum 25-OH-D concentrations (Logan *et al*. 2013). However, there were many drop-outs in the study and a large number of participants were excluded from the analysis on the basis of non-compliance as the data are reported on a per protocol basis rather than on an intention-to-treat basis which increases the risk of bias (Logan *et al.* 2013). Only 13/31 participants randomised to D_2 were included in the analysis

compared with 23/32 randomised to D_3; this puts the study at high risk of bias. In contrast to the findings of Logan *et al*., Biancuzzo *et al. (*Biancuzzo *et al.* 2010*)*, Holick *et al.* (Holick *et al*. 2008) and the present study (Fisk *et al*. 2012) all showed no difference between D_2 and D_3. These studies differ from the conclusions of the meta-analysis (Tripkovic *et al*. 2012). However, the meta-analysis as discussed included assessments using immunoassays that systematically underestimate 25-OH-D_2 as pointed out by Glendenning *et al.* (2013). A definitive answer as to whether there are meaningful differences between D_2 and D_3 in their capacity to raise 25-OH-D concentrations would need a much larger trial with a sample that is more representative of the general population.

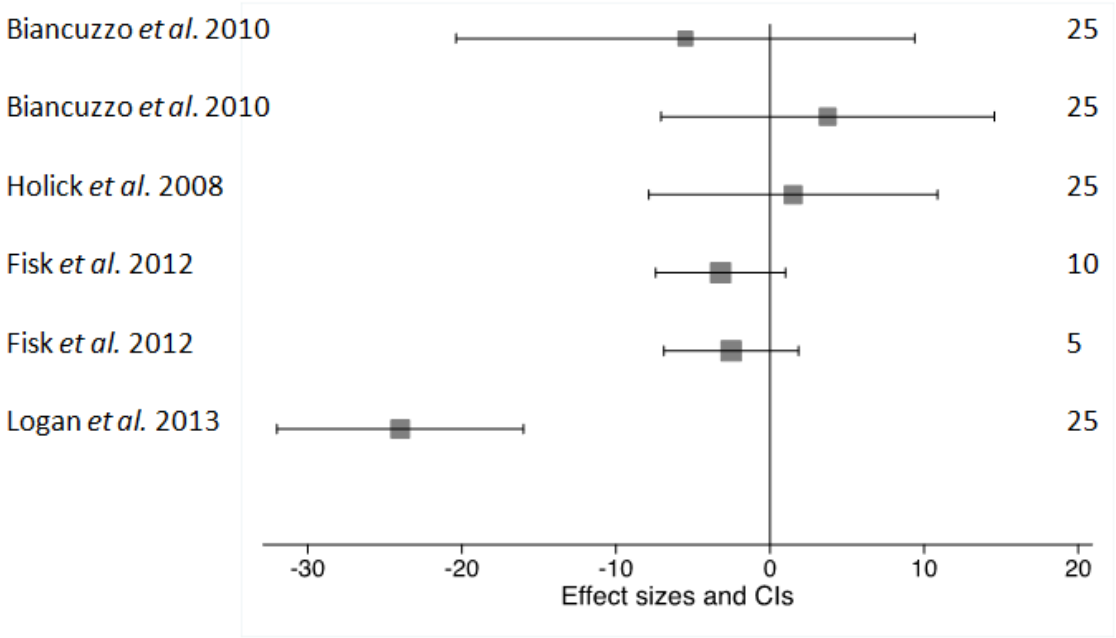


Figure 7.1 Placebo-controlled trials (Biancuzzo *et al.* 2010; Holick *et al.* 2008; Fisk *et al.* 2012; Logan *et al*. 2013) comparing the effect of vitamins D_2 and D_3 at doses ranging from 5-25 µg/d (numbers on right hand side) on total 25-OH-D concentrations measured by HPLC/tandem mass spectrometry. A negative effect size favours vitamin D_3.

It is notable that the increment in 25-OH-D of approximately 2 nmol/L for each µg consumed of D_2 or D_3 in the studies reported in this thesis is greater than the 1.2 nmol/L per µg vitamin D consumed (95% CI 0.72, 1.68) reported in a meta-analysis of 16 RCTs of vitamin D-fortified foods (Black *et al.* 2012). However, the studies included

in the meta-analysis had a high level of heterogeneity including baseline serum 25-OH-D concentrations ranging from 24-84 nmol/L and daily doses of between 3 and 25 µg provided by the foods, therefore results need to be interpreted with caution.

7.1 Associations of vitamin D status with measures of vascular function, inflammation and established CVD risk factors

7.1.1 Arterial stiffness

A strongly significant linear trend for decreasing PWV measured using SphygmoCor with increasing serum 25-OH-D categories was found in the MARINA and CRESSIDA studies in Chapter 3 (P=1.41 x 10^{-6}). This significant association between vitamin D status and arterial stiffness is in agreement with other cross-sectional studies (Al Mheid *et al.* 2011; Giallauria *et al.* 2012; Lee *et al.* 2012; Pirro *et al.* 2012; Seker *et al.* 2013; Webb *et al.* 2012; Zagura *et al.* 2011; Mayer *et al.* 2012; Kuloglu *et al.* 2013), for example, Mayer *et al*. measured PWV using the Sphygmocor device in 560 subjects and found a significantly higher PWV in subjects in the bottom quartile (9.04 m/s) of serum 25-OH-D compared to subjects in the 2^{nd}-4^{th} quartiles (8.07 m/s, 7.93 m/s and 7.70 m/s, respectively) (Mayer *et al.* 2012). A meta-analysis has suggested that even a 1 m/s difference in PWV translates into a 15% difference in CVD risk (Vlachopoulos *et al.* 2010). The results of the present study are summarised with other studies providing a comparison between a higher and lower vitamin D status in **Figure 7.2**. All studies showed a significantly greater PWV at lower concentrations of serum 25-OH-D. Although the finding in Chapter 3 does not prove causation, a variety of mechanisms have been proposed to explain why vitamin D should favourably effect arterial stiffness and these include inhibition of the renin-angiotensin system (Li *et al.* 2002; Li 2003), protection against vascular calcification (Watson *et al.* 1997) or suppression of vascular smooth muscle cell proliferation (Chen *et al.* 2010).

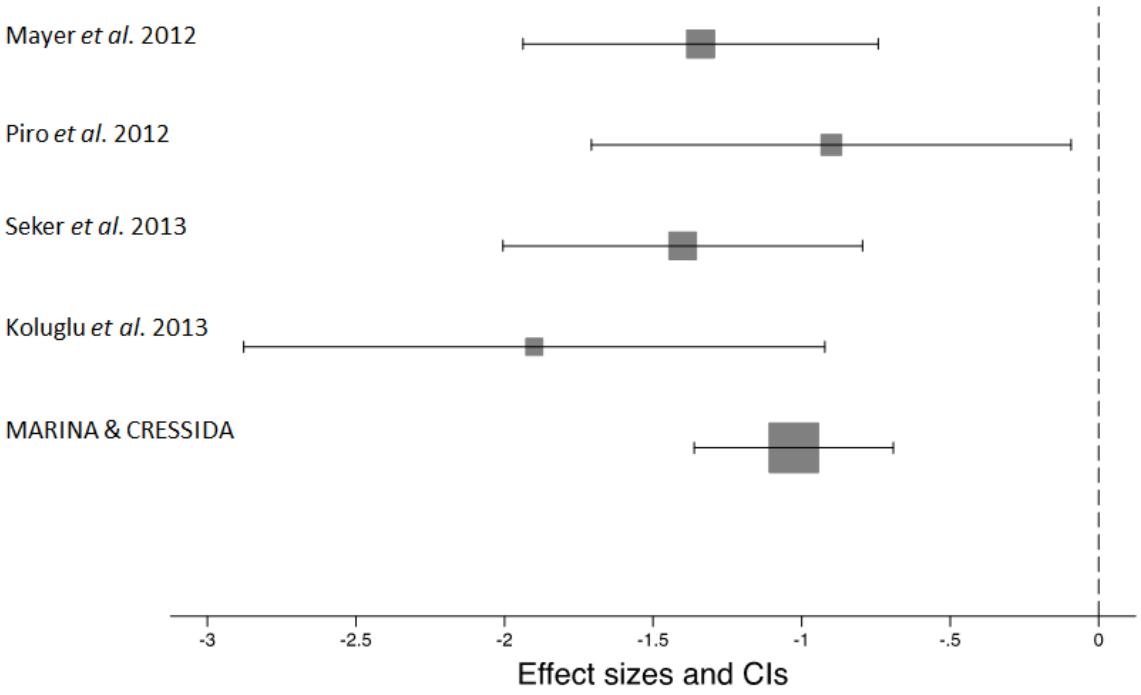


Figure 7.2 Cross-sectional studies comparing participants with higher serum 25-OH-D concentrations to those with lower concentrations. A negative size effect indicates a lower PWV.

PWV measured using the Vicorder device decreased over the 12 wk in the D_2 group compared to baseline in Chapter 5, whereas there was no evidence of a fall in the placebo group. However the difference in the D_2 group compared with placebo did not achieve statistical significance. It seems likely that some of the change in PWV may reflect changes in systolic BP on the D_2 intervention. However, if vitamin D as $1,25(OH)_2D$ prevents the age-related increase in arterial stiffness (Benetos *et al.* 2002) due to inhibition of vascular calcification (Watson *et al.* 1997; de Boer *et al.* 2009), it is likely that a longer term study would be needed to see a change. The few RCTs that have measured arterial stiffness in response to vitamin D supplementation have also all been of a relatively short duration, with the longest lasting 20 wk (Larsen *et al.* 2012). In agreement with the present study, most of these showed no change in PWV (Gepner *et al.* 2012; Larsen *et al.* 2012; Yiu *et al.* 2013). The placebo-controlled studies are summarised in **Figure 7.3**.

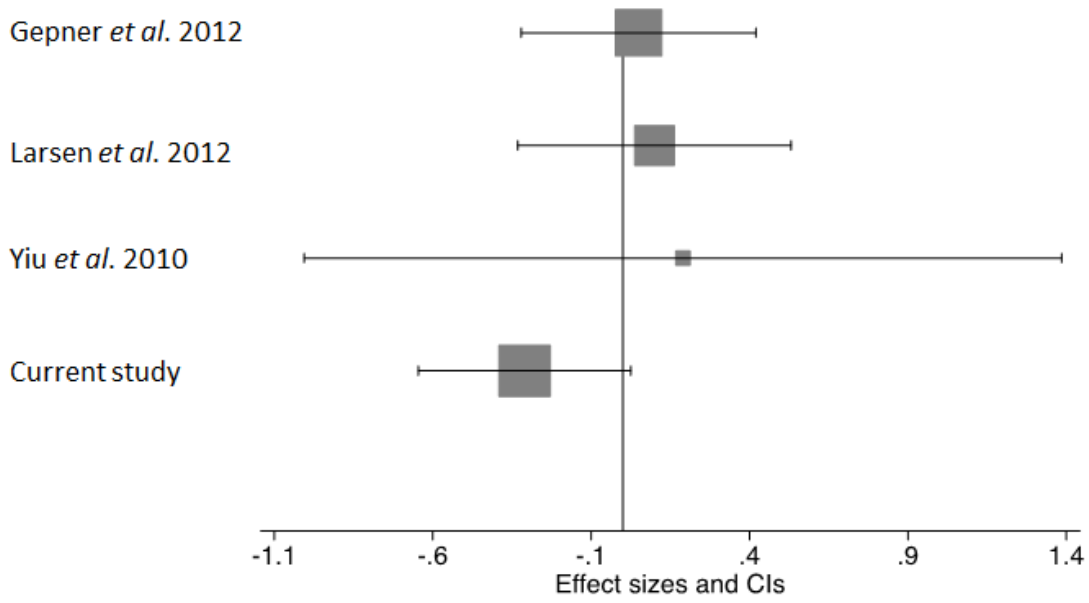


Figure 7.3 Placebo-controlled trials comparing the effect on PWV of daily doses of 62.5 µg D_3 (Gepner *et al.* 2012), 75 µg D_3 (Larsen *et al.* 2012), 125 µg D_3 (Yiu *et al.* 2013) and 10 µg D_2 (current study). A negative size effect indicates a reduction in PWV compared with placebo.

7.1.2 Blood pressure

The most salient finding of the vitamin D_2 intervention was the 4.3 mm Hg lower SBP measured using ambulatory monitoring (P=0.007). As a 20 mm Hg difference in usual SBP is associated with a doubling of risk in CVD mortality (Lewington *et al.* 2002), a 4 mm Hg difference in SBP would be predicted to reduce the risk of CVD mortality by 20%. Whilst some meta-analyses of RCTS have concluded that there is no significant effect of vitamin D on SBP and DBP (Pittas *et al.* 2010; Elamin *et al.* 2011; Kunutsor *et al.* 2014), these have predominantly included studies that have measured BP as seated BP. Only a few vitamin D supplementation trials have measured BP using ABP monitors (Judd *et al.* 2010; Larsen *et al.* 2012; Witham *et al.* 2012; Witham *et al.* 2013c; Witham *et al.* 2014). These were conducted in participants who had hypertension (Larsen *et al.* 2012; Judd *et al.* 2010; Witham *et al.* 2013c; Witham *et al.* 2014) or had previously suffered from a stroke (Witham *et al.* 2012), and none showed a significant change in BP. The finding in the present study of no change in heart rate after vitamin D supplementation suggests that vitamin D may be having an effect on systemic vascular resistance (SVR). There was no treatment effect on FMD implying that the change in BP

was not due to changes in NO production, and there was no change in renin concentrations, suggesting that the effects are independent of the renin-angiotensin system. There is the possibility that changes occurred due to a decrease in PTH concentrations, but the change in PTH was not significant. Therefore, it is most likely that vitamin D was having either a direct effect on vascular smooth muscle cells or an indirect on the central nervous system (CNS) leading to changes in sympathetic stimulation and tone.

Despite the reduced BP after short-term D_2 supplementation in older adults in the RCT conducted as part of this thesis, it cannot be assumed that the findings can be extrapolated to other populations including younger adults and different ethnicities. Older people, for example are more likely to have a high BP (National Institute for Health and Clinical Excellence 2011) and therefore might have a different response to younger adults who have a more healthy BP. Furthermore, different ethnicities in the UK, particularly South Asians have much lower 25-OH-D concentrations compared to the white population (Kift *et al.* 2013), and also a higher risk of hypertension (Whitty *et al.* 1999), so may show an even greater improvement in BP with vitamin D supplementation. It is also not known what effect longer-term, chronic supplementation would have and whether D_3 supplementation would have a similar effect to D_2. A follow-on RCT is needed to determine whether the finding of a reduction in 24 h BP can be replicated in other populations and different ethnicities. This study would be of a larger size and a longer duration of a few years to investigate whether the effect is sustained and also whether arterial stiffness is reduced over a longer period of time.

7.1.3 Indices of inflammation and thrombotic risk

HsCRP and fibrinogen concentrations did not change with vitamin D_2 supplementation, but MMP-9 fell by 19.6% (P=0.035) in the D_2 group compared with placebo. Although MMP-9 was a secondary outcome and there were multiple primary outcomes which increases the probability of this occurring at P=0.05 by chance, this is consistent with the findings of another RCT which randomly allocated 100 type 2 diabetes patients to receive a plain yoghurt drink containing 170 mg calcium and no vitamin D/250 mL or a vitamin D_3-fortified yoghurt drink containing 170 mg calcium and 12.5 µg D_3/250 mL

twice a day for 12 wk and found a significantly greater decrease (*P*=0.005) in MMP-9 concentrations in the D_3 group (-2.3 ± 3.7 ng/mL) compared with placebo (0.44 ± 7.1 ng/mL) (Shab-Bidar *et al.* 2011). There are however few vitamin D supplementation trials that have measured MMP-9 and further research is needed in larger, well-conducted RCTs in order to confirm this finding, although *in vitro* studies have indicated that vitamin D in its active form $1,25(OH)_2D$ down-regulates and suppresses MMP-9 production (Bahar-Shany *et al.* 2010; Anand and Selvaraj 2009; Coussens *et al.* 2009). MMPs provide a continuous source of extracellular matrix degradation within atherosclerotic lesions, contributing to the disruption of the fibrous cap which may lead to plaque rupture. Therefore, if vitamin D supplementation does lead to a decrease in MMP-9 concentrations, it may help prevent the progression of atherosclerosis and plaque rupture (Ferroni *et al.* 2003). MMP-9 might also lead to increased arterial stiffening over time via degradation of elastin (Yasmin *et al.* 2005) and vitamin D supplementation may influence CVD risk by helping to reduce this undesirable effect.

The RCT did not find any significant effects on $FVII_c$. This is consistent with a cross-sectional study which found no significant association between $FVII_c$ and either serum 25-OH-D or $1,25(OH)_2D$ concentrations (Jorde *et al.* 2007).

7.1.4 Serum lipids, fasting glucose and indices of insulin secretion and sensitivity

Changes in lipid concentrations were observed in the RCT after D_2 supplementation; HDL-C, LDL-C and TC all increased significantly compared with placebo. However, there was no effect on the TC:HDL-C ratio which is regarded as the most robust lipid marker of risk (Lewington *et al.* 2007). The LDL-C elevating effect is in agreement with a meta-analysis of 10 vitamin D RCTs which found LDL-C to increase following supplementation (Wang *et al.* 2012a), although the size effect was very small (0.08 mmol/L (95% CI, 0.01, 0.15). The meta-analysis observed no effect for TC and HDL-C apart from in studies lasting longer than 1 y for which there was a significant change in HDL-C concentrations, although this was in the opposite direction to the present study (-2.01 mg/dl, -3.83 to -0.18).

The participants in the present study were predominantly normoglycemic, and vitamin D supplementation did not affect β cell function and insulin sensitivity in agreement with a meta-analysis of 15 vitamin D supplementation (George *et al.* 2012).

7.2 Vitamin D and cognitive function

This thesis found no effect of vitamin D_2 supplementation in 50-70 y olds on measures of mood or cognition. This finding is in agreement with other trials that have measured mood-related outcomes (Dean *et al.* 2011; Dumville *et al.* 2006; Harris and Dawson-Hughes 1993) and cognition (Dean *et al.* 2011; Przybelski *et al.* 2008) and it suggests that there is unlikely to be any benefit on cognitive function of supplementation in the general population. However, studies are needed to investigate whether there is any effect in older adults over 70 years who may have experienced greater cognitive decline.

7.3 Risks and benefits of obtaining vitamin D from food, fortified foods, dietary supplements and sunlight/UVB irradiation

Whilst exposure to UVB irradiation is the most effective means of increasing vitamin D status, it is associated with a major risk of skin cancer (Ashwell *et al.* 2010). Many authorities now advise restricting exposure to sunlight and promote the use of UVB filters in cosmetics and sun lotions which decrease the capacity to synthesise vitamin D (Matsuoka *et al.* 1987). In older age, there is a decreased ability of the skin to produce vitamin D in response to UVB exposure (MacLaughlin and Holick 1985), and older people may be less likely to spend time outdoors due to a lower mobility. Clothing and headscarves block UVB radiation, and UVB exposure is low in the UK winter months. Therefore dietary intake is important for maintaining an adequate vitamin D status in certain at-risk groups and during the winter months.

Dietary intake of vitamin D was low in the studies reported in this thesis; median values (IQR) were 2.3 (1.8-3.8) μg/d in the D_2/D_3 study, 2.6 (2.2-3.7) μg/d in the older population in the second RCT, and 2.7 μg/d for the CRESSIDA and MARINA studies combined. These values are consistent with the most recent NDNS findings from food sources of only of 2.5 μg/d in men (*n*=1126) and 2.1 μg/d (*n*=1571) in women aged 19-64 y (Bates *et al.* 2014). Although there are currently no dietary recommendations for

vitamin D in adults, those confined indoors have a RNI of 10 µg/d (Scientific Advisory Committee on Nutrition 2007). It has been suggested that intakes of 9 µg and 28 µg vitamin D/d would be needed to maintain winter 25-OH-D concentrations in 97.5% of the population above 25 nmol/L or 50 nmol/L respectively, in 97.5% of the population (Cashman *et al.* 2008; Cashman *et al.* 2009). Such intakes are only likely to be achieved through fortification or supplementation.

Strong correlations between serum 25-OH-D concentrations and dietary intake assessed using 4-d food diaries and FFQs were found in the cross-sectional analyses in Chapter 3 and the data presented in this thesis have shown that increasing oily fish intake can significantly increase vitamin D status. In the CRESSIDA study, a group following UK dietary guidelines (DG) including advice to consume 1-2 portions of oily fish/wk for 12 wk were compared to a control group based on a conventional British dietary pattern which included less than 1 serving/month of oily fish. Mean vitamin D intake increased from 3.0 µg/d to 6.6 µg/d in the DG group and the percentage of participants with serum 25-OH-D concentrations >50 nmol/L increased from 60 to 71% over the 12 wk period; concentrations at 12 wk *vs* control were 9.2 nmol/L (95% CI 4.2, 14.2, $P<0.001$) greater. However, diet alone may be insufficient to meet needs in all people and consideration needs to be given to supplementation and fortification of foods.

Supplementation requires remembering to take the supplement on a regular basis or having it regularly administered, but it can be an effective targeted intervention that is possible to adapt to an individual's needs. Massive dosage typically by intramuscular injection can also be a remedy for low vitamin D status in at-risk groups although it does require medical personnel to administer the injection. One possibility is that it could be done at the same time as the influenza vaccination which is offered to older people in the UK and other high risk groups. Fortification of food can be a very effective means of delivering vitamin D providing the food is consumed regularly by a large proportion of the population. Whilst it may not result in a sufficient status (> 75 nmol/L), the RCT in Chapter 4 has shown that even low dose vitamin D provided in a fortified malted milk drink at 5 or 10 µg/d is able to significantly increase serum 25-OH-D concentrations. However, the potential hazards of inducing toxicity in a small

minority has to be considered when fortifying foods. Although an upper level has not yet been established in the UK due to a lack of data, 25 μg/d has been considered as safe and unlikely to cause adverse effects in the general population (Bassuk and Manson 2009). This is low though compared with the tolerable upper intake level of 50 μg/d in North America and Europe (IOM 1997; European Commission Health Consumer Protection Directorate-General 2002). One approach to assess the risks and benefits of fortification is to use that employed by the EU Benefit-Risk Analysis of Foods (BRAFO) project which included an estimation of the risk and benefits of universal folic acid fortification of flour/bread (Verhagen *et al.* 2012).

At present, foods are fortified with either D_2 or D_3, although most often D_3 is used. As the forms seem to be equipotent at low doses, D_2 is likely to be important for widespread fortification as it is more acceptable to vegetarians, vegans and religious groups who may not consume D_3 due to it being from animal sources (Excellence 2014). In the UK, foods tend to be fortified with small quantities based on the serving size, for example, breakfast cereals contain approximately 1.3 μg per 30 g portion and margarine 0.75 μg for every 2 tsp. In the US, vitamin D is added to milk at 10 μg per quart (1 quart = 946.4 mL) (PMO 2007). Based on the results of the RCT, these amounts are unlikely to have much effect on vitamin D status unless the foods are consumed in large quantities. Whilst the current levels of fortification in the UK do make a difference to vitamin D status as shown in the most recent NDNS in adults aged 19-64 where 19% of vitamin D in the diet was coming from fat spreads and 13% from cereals and cereal products, fortification at higher levels and in a product which is consumed widely in a high proportion of the population may be needed to help bring a greater proportion of the population to an adequate vitamin D status. The present study demonstrated that a malted milk drink is an effective vehicle for vitamin D, but a product such as milk which is consumed extensively may be more effective. Many brands of soya milk currently fortify with around 0.75 μg vitamin D/100g. However, data is lacking on the bioavailability of vitamin D_2 and D_3 in different matrices and there is a need for further research in this area.

7.4 Conclusion

One of the main findings of this thesis is that poor vitamin D status is associated with arterial stiffness in healthy older people; this would be predicted to lower CVD mortality risk by 18%. However, it was not possible to show significant changes in arterial stiffness in a short-term trial of vitamin D supplementation; a longer period of intervention is probably necessary to demonstrate major changes in arterial stiffness. The work presented in this thesis also shows that dietary intake has a significant impact on vitamin D status, especially the consumption of oily fish. A dietary intervention with 10 μg D_2/d in the winter months lowered systolic BP by 4.3 mm Hg in older predominantly normotensive adults in the UK winter months, and this change if replicated would be predicted to result in a 20% reduction in the risk of CVD mortality.

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Appendices

APPENDIX 1 Publication in Journal of Nutrition from Chapter 4

The Journal of Nutrition
Nutrient Requirements and Optimal Nutrition

Fortified Malted Milk Drinks Containing Low Dose Ergocalciferol and Cholecalciferol Do Not Differ in Their Capacity to Raise Serum 25-Hydroxy-Vitamin D Concentrations in Healthy Men and Women Not Exposed to UV-B[1–4]

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Abstract

Uncertainly remains regarding the efficacy of low intakes of ergocalciferol (vitamin D2 or D2) and cholecalciferol (vitamin D3 or D3) provided in food to increase serum 25-hydroxy-vitamin D (25-OH-D) metabolite concentrations when UV-B exposure is low. We recruited 40 healthy men and women into a double-blind, parallel design, randomized controlled trial. Participants received placebo or 1 of 4 experimental treatments (D2 or D3 at 5 or 10 μg/d) supplied as a malted milk drink for 4 wk during a period of minimal UV-B exposure in the UK. The primary outcome was a change in serum 25-OH-D2 and 25-OH-D3 measured by ultra-performance liquid chromatography tandem MS. The secondary outcomes were changes in plasma parathyroid hormone and serum calcium (Ca^{2+}). Baseline concentrations (geometric mean ± SD) of 25-OH-D2, 25-OH-D3, and total 25-OH-D were 3 ± 4, 32 ± 22, and 37 ± 22 nmol/L, respectively. Both D2- and D3-fortified drinks resulted in dose-dependent increases ($P < 0.001$) in their respective 25-OH metabolites that did not significantly differ in size. Increments from baseline compared with the placebo group following 5 and 10 μg/d of D2 were (mean ± SEM) 9.4 ± 2.5 and 17.8 ± 2.4 nmol/L for 25-OH-D2 and following 5 and 10 μg/d of D3 were 15.1 ± 4.7 and 22.9 ± 4.6 nmol/L for 25-OH-D3, respectively. There was no difference between D2 and D3 groups in the incremental AUC of their respective metabolites. These findings suggest that D2 and D3 are equipotent in increasing 25-OH-D in healthy men and women with negligible UV-B exposure. J. Nutr. doi: 10.3945/jn.111.156166.

Introduction

Vitamin D insufficiency is common in the UK (1) in winter, as evidenced by a marked seasonal fall in plasma 25-hydroxy-vitamin D (25-OH-D)[7] concentrations, with the nadir occurring in February/March. Dietary vitamin D intakes are low (2–3 μg/d) and mainly provided by meat, eggs, and fish with a smaller contribution from a few fortified foods. The decline is attributed to minimal UV-B exposure between November and March in the UK, which is located between 50° and 60°N.

In addition to the well-established adverse effects on bone metabolism, emerging evidence suggests that vitamin D insufficiency may increase the risk of chronic disease (2,3). The 2 dietary sources of vitamin D, ergocalciferol (vitamin D2 or D2) and cholecalciferol (vitamin D3 or D3), have equivalent effects in preventing rickets in chick bioassays (4). Houghton and Vieth (5) have questioned the ability of D2 to raise serum 25-OH-D concentrations as effectively as D3 and have recommended that D2 should no longer be regarded as suitable for supplementation. Furthermore, they argued that a relatively high intake of vitamin D is required to maintain optimal 25-OH-D concentrations. However, this conclusion was based on studies (6–10) that either did not use specific assays for measuring 25-OH-D metabolites (11), had flaws in their design and execution (i.e., lacking a placebo treatment, did not control for UV-B exposure), or used very high doses of vitamin D that are not relevant to dietary intake (12). Consequently, there is currently a need for a well-controlled, dose-response trial of food fortified with D2 and D3 using specific assays for 25-OH-D2 and 25-OH-D3 (13).

[1] Supported by Biotechnology and Biological Sciences Research Council and GlaxoSmithKline (protocol no. RHS00976).
[2] Author disclosures: C. M. Fisk, H. E. Theobald, and T. A. B. Sanders, no conflicts of interest.
[3] This trial was registered at www.controlled-trials.com/ISRCTN24666304 as ISRCTN Reg. no. ISRCTN24666304.
[4] A CONSORT flow diagram is available from the "Online Supporting Material" link in the online posting of the article and from the same link in the online table of contents at jn.nutrition.org.
[7] Abbreviations used: D2, ergocalciferol; D3, cholecalciferol; 5-D2, 5 μg ergocalciferol/d; 5-D3, 5 μg cholecalciferol/d; 10-D2, 10 μg ergocalciferol/d; 10-D3, 10 μg cholecalciferol/d; iAUC, incremental AUC; 25-OH-D, 25-hydroxy-vitamin D; PTH, parathyroid hormone.
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Manuscript received December 2, 2011. Initial review completed January 20, 2012. Revision accepted April 5, 2012.
doi: 10.3945/jn.111.156166.

An intake of 5 μg represents the recommended daily amount for food labeling purposes in the European Union (14) and 10 μg is the amount recommended for the housebound (15). The aim of the present study was to evaluate the effect on serum 25-OH-D metabolites of intakes of 5 and 10 μg/d of D2 and D3 (5-D2, 10-D2, 5-D3, and 10-D3), provided in a malted milk drink, in the winter months when UV-B exposure is minimal.

Participants and Methods

Participants. Healthy men and women (18–65 y) were recruited from among the staff and student population of King's College London, UK in February 2011. Exclusion criteria were seated blood pressure >160/105 mm, BMI <18.5 and >35 kg/m^2, diabetes or chronic renal, liver, or inflammatory bowel disease, recent exposure to high UV-B light since 1 December 2010, intolerance to the study product (lactose, milk protein), use of prescription calcium or vitamin D, unwillingness to restrict consumption of oily fish to no more than 2 portions (98 g/portion)/wk throughout the study, and unwillingness to abstain from nutritional supplements for the duration of the study. Participants attended a screening visit at which height, weight, and blood pressure were measured and a fasting blood sample was taken to confirm normal hematology, liver function, and plasma glucose. Usual vitamin D intake was estimated by using a FFQ used in the European Prospective Investigation into Cancer and Nutrition. This FFQ estimated nutrient intake from the previous 12 mo and was validated for vitamin D intake in the U.K. population (16). The protocol was approved by the South East London Research Ethics Committee 1 (REC reference 10/H0804/91) and all participants gave written informed consent prior to the study.

Study design. A placebo controlled, double-blind, parallel design was used. Participants were randomly allocated by computer to 1 of 5 groups: placebo or 5 or 10 μg of D2 or D3. The intervention was administered as a malted milk drink (Horlicks, GlaxoSmithKline) in 25-g sachets to be diluted with hot water. The participants were required to consume 1 sachet/d for 4 wk. Each 25-g sachet provided: 92 kcal, 2.4 g protein, 18.9 g carbohydrate (of which 9 g was sugar), 0.8 g fat (of which 0.3 g was saturated), and 100 mg sodium. The D2 and D3 contents of the fortified product were analyzed by International Food Network Ltd using HPLC. The D3 content of the sachets was 5.2 μg for the 5-μg sachets and 10.0 μg for the 10-μg sachets. The placebo product was shown to contain no D2 or D3. Due to difficulties measuring D2, the samples were further analyzed by an HPLC analysis developed for food by the Laboratory of Government Chemists PLC. The D2 content of the sachets was 4.8 μg for the 5-μg sachets and 7.5 μg for the 10-μg sachets. The primary outcome was a change in the serum 25-OH-D concentration and specified secondary outcomes were changes in parathyroid hormone (PTH) and serum calcium (Ca^{2+}) concentrations. Sample size calculations were based on a pilot study conducted in 6 healthy volunteers recruited in the month of February in which the mean plasma 25-OH-D concentrations as measured by RIA increased from 60 nmol/L to 70, 78, and 90 nmol/L after 1, 2, and 3 wk, respectively, of supplementation with 10 μg D3 in cod-liver oil (2 capsules/d, each containing 5 μg as specified by the manufacturer, Seven Seas). Repeat measures on unsupplemented participants showed a correlation of 0.91 and the common SD was 17 nmol/L. The SD of the difference between repeat measures taken 1 wk apart was 7.7 nmol/L. A sample size of 8 per treatment group with 2 measures at baseline and a minimum of 2 measures on follow-up was estimated to be able to detect a 10-nmol/L change in serum vitamin D concentrations with $\alpha = 0.01$ (to allow for multiple comparisons) and 90% power.

Biochemical analysis. Blood samples were obtained by venipuncture by a trained phlebotomist twice at baseline and at wk 1, 2, 3, and 4. Blood was collected into a serum separator tube (Becton Dickinson Vacutainer 367954) and allowed to stand at room temperature for 30 min before centrifuging at 1300 × *g* for 15 min. Serum was separated and stored at −80°C pending analysis of vitamin D metabolites and calcium. Blood collected into EDTA (Becton Dickinson Vacutainer 367836) was chilled on ice, centrifuged at 4°C at 1300 × *g* for 15 min, and plasma separated within 2 h and stored at −80°C pending analysis for PTH. All analyses for each participant were conducted in the same batch to avoid interassay variation and the analysts were unaware of the treatment allocation.

Serum 25-OH-D2 and 25-OH-D3 concentrations were measured by HFL Sport Science using ultra-performance liquid chromatography tandem MS. Briefly, the assays used hexadeuterated internal standards of 25-OH-vitamin D2 and D3, which are spiked into 100-μL aliquots of serum. This is then treated to remove interfering materials by solid phase extraction before ultra-performance liquid chromatography tandem MS. The assay was fully validated over the range of 2.4–363.5 nmol/L for 25-OH-D2 and 7.5–374.4 nmol/L for 25-OH-D3. It had an inter-assay precision (percentage CV) of 5–8% and accuracies (percentage relevant error) of between 0.5 and 6.6% for both metabolites. Liver function tests, blood counts, and blood glucose were determined by KingsPath Clinical Diagnostic Pathology Services at King's College Hospital (London, UK) using routine methods. Calcium was measured using the o-cresolphthalein complexone method on an Advia 2400 analyzer (Siemens Healthcare Diagnostics). Intact PTH was measured using a sandwich chemiluminescent immunoassay on an Advia Centaur analyzer (Siemens Healthcare Diagnostics). This assay has an inter-assay precision (percentage CV) of 3–5%.

Statistical analysis. Statistical analyses of the data were conducted using IBM SPSS for Windows version 20. Where values for 25-OH-D2 were below the limit of detection (2.4 nmol/L), a value of 2.4 nmol/L was assigned. Standard distributional checks were made and where appropriate, analyses were attempted following log or other transformation. Data were analyzed on an intention-to-treat basis by repeated-measures ANOVA (4 time points) with the baseline value as a covariate and treatment group as the between-subjects factor. The analyses were also conducted including gender, but there were no significant gender effects or interactions. Repeated-measures contrasts were performed on the data to test for a dose response and compare the 5- and 10-μg groups in the changes in 25-OH-D metabolite concentrations from baseline to post-intervention. The incremental AUC (iAUC) was calculated using the trapezoid rule. Comparison of the iAUC and changes at wk 4 from baseline were compared by 1-way ANOVA and Dunnett's test was used to indicate a significant difference compared with placebo. All values in the text are mean ± SD unless otherwise stated.

Results

Forty participants (n = 16 males, n = 24 females) were randomly and equally allocated into the 5 treatment groups (**Supplemental Fig. 1**). There were no significant differences across treatment groups in age, vitamin D intake as assessed by FFQ, 25-OH-D concentrations, PTH levels, or Ca^{2+} concentrations (**Table 1**). The mean baseline concentrations of 25-OH-D2, 25-OH-D3, and total 25-OH-D were 3, 38, and 42 nmol/L, respectively, and 27.5, 67.5, and 90.0% of participants were classified as being below the cutoffs of 25, 50, and 75 nmol/L, respectively. One female disliked the test drink and withdrew from the study. Data were available for analysis on 39 participants, who reported consuming all 28 sachets.

Serum vitamin D metabolite concentrations remained low and unchanged in the placebo group. The 25-OH metabolite concentrations rose steadily in the first 3 wk of supplementation and appeared to have reached a plateau at 4 wk because there was no evidence of further increases between wk 3 and 4 (**Fig. 1**). There was a significant dose-response relationship between the changes in serum analyte concentrations. Repeated-measures contrasts showed greater increases in 25-OH-D2 over the 4 wk in the 10-D2 group compared with the 5-D2 group (P = 0.007). Similarly, the increase in 25-OH-D3 in the 10-D3 group was greater than that in the 5-D3 group (P = 0.003). D2 did not

TABLE 1 Baseline characteristics of participants at randomization by treatment allocation[1]

Characteristics	Placebo	5-D2	5-D3	10-D2	10-D3
Male : female	3 : 5	3 : 5	3 : 5	3 : 5	5 : 3
Age, *y*	24.1 (1.8)	24.4 ± 4.7	30.5 ± 11.0	24.4 ± 3.9	30.6 ± 10.6
White : non-white, *n:n*	6 : 2	6 : 2	6 : 2	8 : 0	5 : 3
BMI, *kg/m*^{2}	21.3 (2.1)	20.4 ± 1.5	25.7 ± 2.8	22.9 ± 2.7	24.8 ± 3.9
Vitamin D intake,[2] *μg/d*	2.4 (1.6)	2.6 ± 1.3	2.4 ± 1.3	3.2 ± 1.3	2.6 ± 1.2
Calcium intake, *mg/d*	902 (346)	806 ± 223	880 ± 249	1000 ± 220	1019 ± 279
Serum 25-OH-D2, *nmol/L*	3.2 ± 1.2	3.8 ± 8.2	2.6 ± 0.7	2.7 ± 0.6	2.5 ± 0.1
Serum 25-OH-D3, *nmol/L*	29.7 ± 13.7	41.3 ± 27.6	28.1 ± 22.1	39.0 ± 14.1	28.0 ± 29.1
Total serum 25-OH-D, *nmol/L*	33.5 ± 13.3	48.0 ± 26.6	31.3 ± 22.1	41.9 ± 14.1	30.9 ± 29.1
Serum Ca^{2+}, *mmol/L*	2.25 ± 0.07	2.31 ± 0.05	2.28 ± 0.05	2.27 ± 0.07	2.25 ± 0.07
Plasma PTH, *ng/L*	48.0 ± 10.1	44.9 ± 9.2	40.9 ± 11.7	45.7 ± 9.9	47.0 ± 15.1

[1] Values are means ± SD or geometric mean (SD). Variables did not differ among the groups. D2, ergocalciferol; D3, cholecalciferol; 5-D2, 5 μg ergocalciferol/d; 5-D3, 5 μg cholecalciferol/d; 10-D2, 10 μg ergocalciferol/d; 10-D3, 10 μg cholecalciferol/d; 25-OH-D, 25-hydroxy-vitamin D; PTH, parathyroid hormone.
[2] Includes both D2 and D3 from fortified foods.

affect the concentration of 25-OH-D3, nor did D3 affect that of 25-OH-D2. The iAUC (nmol · L^{-1} · wk) for 25-OH-D2 in the D2 groups (5-D2 = 24 ± 11; 10-D2 = 45 ± 26) were not significantly different from those for 25-OH-D3 in the D3 groups (5-D3 = 23 ± 13; 10-D3 = 49 ± 25). The total 25-OH-D concentration differed from the placebo group in the 5-D2, 10-D2, 5-D3, and 10-D3 groups ($P \leq 0.001$). Furthermore, the iAUC (nmol · L^{-1} · wk) for total 25-OH-D in the D2 groups (5-D2 = 15 ± 15; 10-D3 = 35 ± 27) did not differ from those in the D3 groups (5-D3 = 23 ± 14; 10-D3 = 49 ± 24).

Compared with placebo, the increment in serum 25-OH-D2 from baseline to postintervention was greater in the 5-D2 and 10-D2 groups (both $P < 0.01$), as was the increase in 25-OH-D3 greater in the 5-D3 and 10-D3 groups ($P < 0.05$ and $P < 0.01$) (**Table 2**). The increment in serum total 25-OH-D did not differ between the D2 and D3 supplement groups. There was no significant difference between treatment groups in the change in serum Ca^{2+} concentrations from baseline to postintervention. Although there was a decrease in PTH of 13 ng/L (95% CI: −21, −3) in the 10-μg/d groups, this change did not differ from that in the placebo group [7 ng/L (95% CI: −21, 7)].

Discussion

This study set out to compare low intakes of D2 with D3, as might be provided by fortified foods, on serum vitamin D metabolite concentrations. The study addresses an important question regarding the efficacy of fortified foods in helping to maintain normal serum 25-OH-D concentrations. At baseline, 67.5% of participants had a 25-OH-D concentration <50 nmol/L. This level is generally regarded as representing the lower limit of adequacy in vitamin D status. After a month of 5 or 10 μg/d of D2 or D3, 35% of participants in the treatment groups continued to have values <50 nmol/L. The study is particularly relevant to the UK because of the relatively large population of vegetarians, particularly those of South Asian origin. D2 is derived from fungi and is acceptable to people unwilling to consume animal products, whereas D3 is derived from animal products. This study was conducted at a time of year when UV-B exposure is extremely limited in the UK. We also took the precaution of excluding participants who had recent high exposure to UV-B. Most participants did not consume any D2. However, in one participant, we found high (>25 nmol/L) concentrations of 25-OH-D2 in serum at baseline that were later found to be derived from fortified soya milk. Nevertheless, we were able to show a very clear dose-response relationship between D2 intake and serum 25-OH-D2 and there was considerably less variability in this analyte than that of 25-OH-D3, which is influenced by UV-B exposure as well as dietary intake. There was no evidence of any change in 25-OH-D3 concentrations in the participants allocated to placebo or the D2 groups, confirming that the results were not confounded by variations in exposure to UV-B during the study.

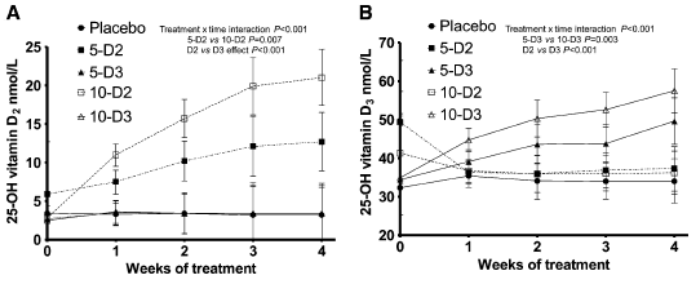

FIGURE 1 Serum concentrations of 25-OH-D2 (*A*) and 25-OH-D3 (*B*) in healthy men and women not exposed to UV-B following placebo, 5-D2, 5-D3, 10-D2, and 10-D3 for 4 wk in the winter months. Values are geometric mean and 95% CI, $n = 8$ or 7 (5-D3). Log-transformed data were analyzed by repeated-measures ANOVA (4 time points) with the baseline value as a covariate and treatment group as the between-subjects factor. Repeated-measures contrasts were performed to test for a dose-response effect. 5-D2, 5 μg ergocalciferol/d; 5-D3, 5 μg cholecalciferol/d; 10-D2, 10 μg ergocalciferol/d; 10-D3, 10 μg cholecalciferol/d; 25-OH-D, 25-hydroxy-vitamin D.

TABLE 2 Changes from baseline in concentrations of serum 25-OH-D metabolites and calcium and plasma PTH after 4 wk of consuming a drink providing a daily intake of 5μg or 10μg of D2 or D3 or placebo in participants not exposed to UV-B[1]

Analyte	Placebo	5-D2	5-D3	10-D2	10-D3	*P* value[2]
Δ25-OH-D2, *nmol/L*	−0.2 (−1.0, 0.5)	9.2 (5.5, 12.9)**	−0.1 (−0.2, 0.1)	17.6 (9.2, 25.9)**	−0 (−0.1, 0.1)	<0.0001
Δ25-OH-D3, *nmol/L*	−3.1 (−8.0, 1.7)	−3.8 (−10.3, 2.8)	12.0 (2.7, 21.2)*	−2.9 (−9.3, 3.4)	19.8 (9.4, 30.2)**	<0.0001
ΔTotal 25-OH-D, *nmol/L*	−3.4 (−8.2, 1.5)	4.9 (−2.3, 12.7)	11.9 (2.7, 21.2)*	13.6 (4.1, 23.0)**	19.7 (9.4, 30.1)**	0.001
ΔCa^{2+}, *mmol/L*	−23 (−54, 99)	−39 (−145, 67)	−17 (−87, 57)	−15 (−85, 123)	−21 (−85, 124)	0.728
ΔPTH, *ng/L*	−6.6 (−22.0, 9.7)	3.8 (−18.1, 25.7)	2.4 (−20.0, 24.9)	−10.6 (−10.0, −1.2)	−15.7 (−15.7, 2.6)	0.269

[1] Values are mean change from baseline (95% CI), $n = 8$ or 7 (5-D3). Asterisks indicate different from placebo: *$P < 0.05$; **$P < 0.01$. 5-D2, D2, ergocalciferol; D3, cholecalciferol; 5-D2, 5 μg ergocalciferol/d; 5-D3, 5 μg cholecalciferol/d; 10-D2, 10 μg ergocalciferol/d; 10-D3, 10 μg cholecalciferol/d; 25-OH-D, 25-hydroxy-vitamin D; PTH, parathyroid hormone.
[2] *P* value from 1-way ANOVA of the changes from baseline between treatment groups.

The size of the increment in the respective 25-OH metabolite concentrations did not differ between D2 and D3 treatments. This would indicate that both forms of the vitamin are equipotent. Much of the uncertainty regarding the ability of the 2 forms to increase vitamin D status has arisen because of the use of nonspecific assays. Five previous studies concluded that D2 is less effective than D3 at increasing serum 25-OH-D concentrations (6–10), but these were all uncontrolled and 3 used an assay that was not able to measure 25-OH-D2 and 25-OH-D3 separately (8–10). All of the studies, apart from the study by Binkley et al. (7), which gave participants 40 μg/d or 1250 μg/wk, also used supplements providing >100 μg. Two had given very large single doses of vitamin D at 7500 μg (9) or 1250 μg (6). It is not possible to extrapolate from such high doses to the low amounts provided in fortified foods. A strength of our study is that we used a highly sensitive ultra-performance liquid chromatography tandem MS assay that separated the 2 vitamin D metabolites. This assay uses only 0.1 mL of serum but has sensitivity over the range of 2.4–363.5 nmol/L for 25-OH-D2 and 7.5–374.4 nmol/L for 25-OH-D3, with accuracies of between 0.5 and 6.6% for both metabolites. To our knowledge, our study is the first to compare the 2 forms of vitamin D at an intake within the dietary range. Our findings are broadly in agreement with a study by Holick et al. (12) in which healthy adults were randomly allocated to receive 25 μg of D3 ($n = 20$), 25 μg of D2 ($n = 16$), 12.5 μg of each of D3 and D2 ($n = 18$), or a placebo once per day for 11 wk. Their measurements were conducted using liquid chromatography and tandem MS. Their detection limit for the assay was 10 nmol/L and the interassay coefficient of variation was ~10%. They found both 25-OH-D2 and 25-OH-D3 to be equally effective. In our study, serum 25-OH metabolite concentrations appeared to reach a plateau at 4 wk, whereas in the study by Holick et al. (12), it was suggested that the plateau was reached after 6 wk. However, closer inspection of their data suggests that later values decreased closer to the 4-wk value. A recent study by Biancuzzo et al. (17) gave participants 25 μg/d of D2 or D3 in orange juice for 11 wk at the end of winter in the US. They similarly showed that a plateau in 25-OH-D2 and 25-OH-D3 was reached after 4–5 wk of supplementation and that both D2 and D3 were equally effective at raising and maintaining 25-OH-D concentrations.

In our study, the mean absolute increases compared with placebo for 25-OH-D2 were 9.4 ± 2.5 and 17.8 ± 2.4 nmol/L following 5-D2/d and 10-D2/d and for 25-OH-D3 were 15.1 ± 4.7 and 22.9 ± 4.6 nmol/L following 5-D3/d and 10-D3/d. This would suggest that for 1 μg of D2 or D3, there is an ~2-nmol/L increase in the 25-OH-D metabolite. For comparison, Holick et al. (12) found that supplementation with 25 μg/d led to serum 25-OH-D increases of 24.7 nmol/L in the D2 group ($n = 16$) and 23.2 nmol/L in the D3 group ($n = 20$) after 11 wk, equivalent to increases in serum 25-OH-D of ~1 nmol/L per μg vitamin D, which is about one-half the value observed in the present study. It is possible that higher intakes become less effective in raising 25-OH-vitamin D concentrations because they increase the catabolic rate of the vitamin. However, the mean baseline 25-OH-D value in Holick's (12) study was 47.0 nmol/L compared with the mean of 42.0 nmol/L at baseline in this study. It may therefore be that a greater treatment response is seen in participants who start with a lower serum 25-OH-D concentration.

Supplementation with D2 did not influence serum concentrations of 25-OH-D3. Previously, Armas et al. (6) reported that D2 supplementation led to a decline in 25-OH-D3 and suggested that this may be due to competition for the 25-hydroxylase by D2 and/or an increased metabolic degradation of 25-OH-D3. However, this study used a RIA and was conducted in July in Nebraska, when UV-B exposure would be high and the average sun exposure was 10 h/wk, whereas we have found that exposure to the equivalent of even 15 min of mid-day sunlight provided by a suberythremal dose of radiation can markedly increase vitamin D concentrations (T.A.B. Sanders, A. Young, S. Walker, unpublished observations). Our findings support those of Holick et al. (12) and provide no evidence to suggest that D2 influences the catabolism of D3 or its metabolites.

We found some tentative evidence for a decline in PTH concentrations with 10 μg/d of vitamin D, but this did not significantly differ from placebo. However, because there is high variability between participants in PTH, it would be necessary to study a much larger number of participants to confirm this effect. It is also possible that due to the adequate calcium intakes at baseline, higher concentrations of serum 25-OH-D may not decrease PTH concentrations any further.

The strengths of this study are that it was conducted in the winter months using a physiologically relevant dose of vitamin D and that we used a highly sensitive and specific assay to measure 25-OH-D2 and 25-OH-D3. The food matrix used in the present study was a drink, which may have facilitated absorption of the vitamin and so the results cannot necessarily be extrapolated to other foods, i.e., breakfast cereals. A limitation of the study is that the participants were in good health and not obese. With increasing age and obesity, there may be an increase in the rate of turnover of vitamin D, resulting in lower serum concentrations.

In conclusion, the present study indicates equivalent bioavailability of D2 and D3 in a malted milk drink. There remains a need for well-controlled, long-term studies of low-dose fortification on clinical endpoints rather than surrogate markers of disease risk.

Acknowledgments

T.A.B., H.E.T., and C.M.F. designed the research; C.M.F. conducted the research; T.A.B. and C.M.F. analyzed the data; and C.M.F. and T.A.B. wrote the paper. All authors read and approved the final manuscript.

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APPENDIX 2 Abstract for the 2014 Nutrition Society Summer Meeting Postgraduate Competition

Vitamin D and Cardiovascular Disease By C.M.Fisk[1], H.E.Theobald[2], P.J.Chowienczyk[3] and T.A.B.Sanders[1], [1]Diabetes and Nutritional Sciences Division and [3]Cardiovascular Sciences Division, King's College London, 150 Stamford Street, SE1 9NH, [2]GSK Consumer Healthcare, GlaxoSmithKline, Brentford, TW8 9GS.

Lower serum 25-hydroxy vitamin D (25-OH-D) concentrations are associated with a greater risk of CVD mortality, coronary heart disease and stroke (1). Besides dietary intake of vitamin D, serum 25-OH-D is also influenced by UVB exposure and vitamin D binding protein concentration. Randomised controlled trials (RCT) have yet to show any clear benefit of vitamin D supplementation on CVD risk or mortality (2, 3), but vitamin D might influence vascular function and inflammation. Vieth *et al* (4) argue that recommended dietary intakes for vitamin D are unlikely to maintain optimal serum 25-OH-D concentrations and that UVB exposure, which increases risk of skin cancer, or high dose supplementation are needed. Most published trials testing the effects on CVD risk factors have used pharmacological doses of vitamin D (5).

We investigated the effect of low dose vitamin D_2 supplementation on CVD risk factors in the winter months, when UVB exposure is minimal. In the first RCT, we showed that both D_2 and D_3 fortified drinks (at two dose levels of 5 or 10 μg/d), taken for 4 wk, resulted in significant dose dependent (P<0.001) increases in their respective 25-OH metabolites, measured by ultra-HPLC tandem mass spectrometry; mean increments ± SED from baseline, compared with the placebo group, following 5 and 10 μg D_2/d were 9.4 ± 2.5 and 17.8 ± 2.4 nmol/L for 25-OH-D_2, and following 5 and 10 μg D_3/d were 15.1 ± 4.7 and 22.9 ± 4.6 nmol/L for 25-OH-D_3 (6). In a second placebo-controlled RCT, which compared effects on CVD risk factors, 41 predominantly normotensive men and post-menopausal women (50-70 y) consumed drinks providing an intake equivalent to 10 μg/d D_2, for 12 weeks in the winter months. The primary outcome was endothelial function measured as flow mediated dilation of the brachial artery; secondary outcomes were 24-h ambulatory blood pressure (BP), arterial stiffness and inflammatory markers; compliance to treatment was determined by measuring vitamin D status. Mean differences ± SED from baseline *vs*. placebo were 20.6 ± 2.9 nmol/L (P<0.001) for 25-OH-D_2 but there were no differences between treatments in 25-OH-D_3. Both 24 h systolic and diastolic BP fell on D_2 treatment compared with placebo: mean differences (95% CI) in mm Hg were -4.2 (-7.3, -1.2; P=0.008) and -2.5 (-5.2, 0.1; P=0.061) respectively. No significant differences in flow mediated dilation, arterial stiffness or inflammatory markers were found. In conclusion, our data suggest that D_2 and D_3 are equipotent in increasing 25-OH-D, and that a dose of 10 μg/d D_2 for 12 weeks has favourable effects on BP compared with placebo in healthy men and women with negligible UV-B exposure.

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APPENDIX 3 Abstract published in Proceedings of the Nutrition Society of work in Chapter 3

Proceedings of the Nutrition Society (2013), **72 (OCE4)**, E188 doi:10.1017/S0029665113002139

Summer Meeting, 15–18 July 2013, Nutrition and healthy ageing

Advice to consume 1–2 portions of oily fish per week improves vitamin D status

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Dietary sources of vitamin D may only play a minor role in meeting vitamin D requirements compared with ultraviolet exposure[(1)]. Data from the National Diet and Nutrition Survey suggest that vitamin D insufficiency, defined as serum 25-hydroxyvitamin D (25(OH)D)<25 nmol/L, is widespread in the UK[(2)]. Except for oily fish, there are few rich dietary sources of vitamin D. Furthermore, high intakes of unrefined cereals can contribute to low serum 25(OH)D concentrations by increasing the catabolism of vitamin D which is thought to be due to their high phytic acid content interfering with calcium absorption[(3)]. We report the association between dietary vitamin D intake and 25(OH)D at baseline in subjects recruited into a randomised controlled trial, and the changes in vitamin D intake and 25(OH)D following randomisation to two dietary interventions.

The CRESSIDA trial (ISRCTN92382106) randomised 165 healthy non-smoking men and women aged 40–70 y to a cardioprotective (**CP**) or a control (**C**) diet for 12 wks. Blood samples and 4-d diet records completed at baseline and follow-up were available for analysis in 162 subjects. The cardioprotective diet included salt <6 g/d, saturated fatty acids <10% of food energy, oily fish intake 1–2 portions/wk and fruit and vegetables 5 portions/d, and supplied at least half of the cereal intake from wholegrains. The control diet was a typical well balanced British diet but contained oily fish less than once a month. All subjects abstained from dietary supplements throughout the study. Table 1 shows vitamin D intakes and plasma concentrations of 25(OH)D determined by immunoassay.

	Vitamin D intake (μg/d)				25(OH)D (nmol/L)			
	CP (n = 80)		**C** (n = 82)		**CP** (n = 80)		**C** (n = 82)	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Baseline	3.0	2.5	2.9	2.3	58.0	18.7	60.3	22.8
Follow-up	6.6*	4.3	2.7	1.4	68.4*	23.0	58.4	19.9

*P<0.001 compared to control group..

Dietary vitamin D intake and 25(OH)D concentrations were correlated at baseline (r = 0.277; P<0.001). Vitamin D intake increased following the cardioprotective diet compared with the control as a consequence of the increased consumption of oily fish. Serum 25(OH)D concentrations at 12 weeks were 9.2 nmol/L (P<0.001) greater in the **CP** diet group compared to the **C** group when adjusted for baseline 25(OH)D, age, BMI, gender, ethnicity and seasonality. This increase is similar to the increase in 25(OH)D found after supplementation with 5 μg vitamin D3 per day for 4 weeks in the winter months[(4)]. In conclusion, the consumption of oily fish 1–2 times a week improves vitamin D status, and the inclusion of wholegrain cereals (mainly breakfast cereals and bread) as part of a cardioprotective diet does not have an adverse effect on vitamin D status.

This work was supported by the Food Standards Agency and Department of Health.

1. Norman A, Bouillon R, Whiting S *et al.* (2007) *J Steroid Biochem Mol Biol*. **103**, 204–205.
2. Bates B, Lennox A, Prentice A *et al.* (editors) (2012) Department of Health. 1–79.
3. Dagnelie PC, Vergote F, van Staveren WA *et al.* (1990) *Am. J. Clin. Nutr.* 51, 202–208.
4. Fisk CM, Theobald HE & Sanders TAB (2012) *J Nutr* 142, 1286–1290.

APPENDIX 4 Ambulatory blood pressure monitor instructions (left arm version)

What to do when driving

- When driving, if a measurement starts, continue to drive normally and do not worry about keeping the arm still (note in the Diary Card if this happens).
- Alternatively turn the monitor off whilst driving (by pressing the black ON/OFF button until the machine beeps and 'A' is no longer displayed). Remember to turn the monitor back on when you arrive!

Can I take a shower or bath?

- When taking a shower / bath, please take the cuff off and do not wear the monitor.
- Ideally try to time your shower or bath so it falls in between measurements or take a shower / bath before starting measurements.

Between measurements

- Engage in normal activities.
- Check the yellow mark on the cuff is still in position over the artery, as the cuff may move during the day.

Once your measurements are finished

When you have finished your 25 hours of measurements please can you:

- Turn off the monitor by pressing the black ON/OFF button for at least 3 seconds until it bleeps and the letter 'A' disappears from the display.
- Ensure your Diary Card has been completed.

If the monitor is being collected by courier:

- Package the completed Diary Card and the monitor, cuff and belt or strap (if supplied) in the provided addressed padded envelope and seal ready for collection.

If you are bringing the monitor in:

- Store and transport the completed Diary Card and the monitor, cuff and belt or strap (if supplied) in the provided padded envelope.
- The monitor can be taken into the Nutritional Sciences Department (4th Floor Franklin-Wilkins Building, Kings College London) and left with Laura O'Sullivan in the Nutritional Sciences Division reception (Room 4.68).

If you are uncertain about what to do or if you experience any difficulties, please contact Catherine Fisk on the phone number on the front of this booklet or by e-mail (drisk@kcl.ac.uk).

KING'S College LONDON

University of London

The effect of low dose vitamin D2 on cardiovascular risk

24 Hour Blood Pressure Monitoring

Instructions for Participants *[Left arm version]*

We are asking you to make this measurement to monitor what is happening to your blood pressure over a 24 hour period as you go about your daily routine and when asleep. We will ask you to make these measurements at screening and on 3 occasions during the study, after each visit.

Please try to make the measurement over 25 hours, as the first hour does not always give a representative reading.

During the day (7am – 10pm), the cuff will inflate every 30 minutes and during the night (10pm-7am) every 1 hour. Please keep your arm still during measurements. Between measurements you can continue with your usual activities.

We will provide you with a Diary Card to complete on measurement days. Please fill in your activity at the time of each measurement, the time you go to bed, the time you get up and any unusual circumstances.

If you have any difficulties or questions, please contact Catherine on:

drisk@kcl.ac.uk
020 7848 4301

THANK YOU!

Fitting the cuff and device

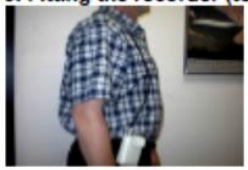
1. Fit the cuff to your arm – please fit to your UPPER LEFT arm

- Wrap cuff around your bare upper left arm (not over your clothes)
- Position lower edge of cuff 1 inch (2 cm) above the inside of the elbow, with the yellow mark placed centrally over the crease in your arm.
  - the crease is on the opposite side to your elbow.
- Wrap the cuff so it fits snugly but loose enough to allow two fingers between the cuff and your arm.

2. Position the air hose connecting the cuff to the recorder

- The air tube should be at the top of the cuff.
- Position the air hose over the shoulder and round the back of your neck to your RIGHT side as shown in the photo

3. Fitting the recorder (to allow you to walk around during the daytime)



- The recorder can either be attached to a belt (either your own or the provided belt) or worn like a bag.
  - If attached to a belt, position the recorder on your RIGHT side.
  - If worn like a bag, place strap over left shoulder and across your body to your RIGHT side.

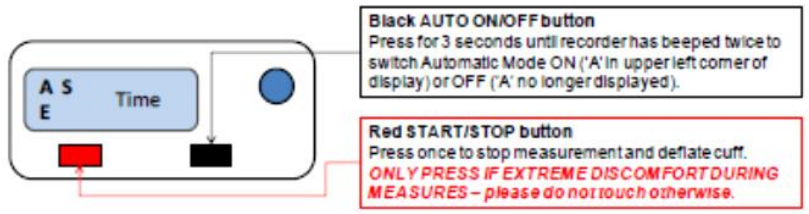
4. Starting and ending the measurements

- To start measurements, press black 'AUTO ON/OFF' button and hold for at least 3 seconds until it has bleeped twice and **'A' appears on the top left corner of the display**.

- When you have finished the 25 hours of measures, turn the monitor off by pressing the black 'AUTO ON/OFF' button and holding for at least 3 seconds until it has bleeped twice and **'A' disappears from the display**.

At night: recorder can be removed from the belt and/or your shoulder and placed on your bedside table. Place recorder on its side to avoid kinks in the air hose.

Some notes on operation and the display



Display
A - Indicates recorder is ON and in Automatic Mode
S - Indicates unit is in SLEEP MEASUREMENT MODE
E – Indicates an ERROR with measurement. Check cuff fitted properly and no kinks in air hose. Monitor will re-try measurement. **Contact us if problem persists.**

Procedure on measurement days

- The monitor has two programs depending on the time of day/night:
  - **DAY SETTING (7am – 10pm)**: The cuff will inflate every 30 minutes on the hour and half hour (i.e. at 7am, 7.30am, 8.00am, etc until 10pm)
  - **NIGHT SETTING (10pm – 7am)**: The cuff will inflate every 1 hour on the hour (i.e. at 10pm, 11pm, 12 midnight, etc until 7am)
- On measurement days, try not to alter your routine - have as normal a day as possible.
- Please try to wear the monitor for at least 25 hours (e.g. from 8am until 9am the following morning), as readings collected during the first hour are not always representative.

Diary Card

We will provide a Diary Card for you to complete on the day of measurement with your activity level at the time of each measurement. Please complete this for each measurement. Please also note what time you go to bed and what time you get up.

During a measurement

- **The arm must be kept still and relaxed during measurements** to avoid incorrect results.
  - **If you are standing:** let your arm hang loosely, whilst keeping it still.
  - **If you are sitting:** rest your arm loosely on a table or hang your arm loosely, whilst keeping it still.
- Please avoid opening and closing your hand or moving your fingers.
- Ensure the air hose is not kinked when a measurement is being taken.

APPENDIX 5 Ambulatory blood pressure monitor diary card for DRISK study

KING'S College LONDON
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The effect of low dose vitamin D2 on cardiovascular risk

Blood Pressure Monitoring
DIARY CARD

Participant ID:
.....

Please complete this diary card when you are wearing the blood pressure monitoring device.

If you experience any pain or an extremely unpleasant sensation during the blood pressure measurements please turn off the unit immediately using the ON / OFF key and remove the cuff.

If you have any difficulties or questions,
please contact Catherine Fisk on:

drisk@kcl.ac.uk

020 7848 4301

THANK YOU!!

Blood Pressure Monitoring Diary

Date started: ...../...../..... **Time started:** .....

Time you go to sleep:..... **Time you wake up next day** :.....

Time of last measurement: .....
Please wear for 25 hours (e.g. from 10.00am 1st day until 11.00am next day)

Did you take any exercise? Yes / No
If Yes, what time(s):
..... Please give further information in the activity diary below

The cuff will inflate every 30 minutes during the day (7am – 10pm) and every hour during the night (10pm-7am). Keep your arm still during measurements. Between measurements you can continue your usual activities.

Activity diary

At the time of each measurement please fill in your activity level and any unusual circumstances. *An example of how to complete the diary is shown in the shaded area of the table.*

Time	Activity level (please circle)				Other
08.30	Lying down	Sitting	Standing	Walking	
09.00	Lying down	Sitting	Standing	Walking	Standing on train
09.30	Lying down	Sitting	Standing	Walking	Sitting at desk
	Lying down	Sitting	Standing	Walking	
	Lying down	Sitting	Standing	Walking	
	Lying down	Sitting	Standing	Walking	
	Lying down	Sitting	Standing	Walking	
	Lying down	Sitting	Standing	Walking	
	Lying down	Sitting	Standing	Walking	

Time	Activity level (please circle)				Other
	Lying down	Sitting	Standing	Walking	
	Lying down	Sitting	Standing	Walking	
	Lying down	Sitting	Standing	Walking	
	Lying down	Sitting	Standing	Walking	
	Lying down	Sitting	Standing	Walking	
	Lying down	Sitting	Standing	Walking	

APPENDIX 6 D_2/D_3 study blood handling protocol at screening and on study visits

SAMPLE	TUBES		ICE/RT	CENTRIFUGE	SEPARATION‡	ANALYTES	STORAGE	TRANSPORT
Screening (11mL)	2 mL Fluoride oxalate (grey)	BD Vacutainer 368920	Room temperature	15 min x 1300 g @ 4˚C	Separate plasma into 2 x 0.4 mL aliquots	Glucose	Freeze 1 aliquot and send the other fresh to KCH	Same day to KCH at room temperature
	2 mL EDTA (lavender)	BD Vacutainer 367836	Room temperature	No	None	FBC	Room temperature	Same day to KCH at room temperature
	2 mL EDTA (lavender)	BD Vacutainer 367836	Ice	15 min x 1300 g @ 4˚C	Separate plasma into 2 x 0.4 mL aliquots within 2h	PTH	Freeze both aliquots and store at -80°C	Store and transport later in batches to KCH in dry ice
	5mL No anticoagulant (gold)	BD Vacutainer 367954	Stand for 30 min at room temperature before centrifuging	15 min x 1300 g @ 4˚C	Separate into 1 x 1 mL and 2 x 0.5mL aliquots	LFT, Calcium, 25-OH-D2 and 25-OH-D3	Freeze both 0.5mLaliquots and send the 1mL fresh to KCH. Keep the vit D sample to send to HFL at the end of the study.	Same day to KCH at room temperature (for LFT and Calcium). Transport vit D samples to HFL Sport Science in dry ice at end of the study.
Baseline and Visit 4 (7mL)	2 mL EDTA (lavender)	BD Vacutainer 367836	Ice	15 min x 1300 g @ 4˚C	Separate plasma into 2 x 0.4 mL aliquots within 2h	PTH	Freeze both aliquots and store at -80°C	Store and transport later in batches to KCH in dry ice.
	5mL No anticoagulant (gold)	BD Vacutainer 367954	Stand for 30 min at room temperature before centrifuging	15 min x 1300 g @ 4˚C	Separate into 1 x 1 mL and 2 x 0.5mL aliquots	Calcium, Albumin, 25-OH-D2 and 25-OH-D3	Freeze all aliquots and store at -80°	Keep vitamin D samples – send all together to HFL Sport Science at the end of the study. Store Ca/Albumin sample at -80° and transport later in batches.
Visits 1,2 and 3 (5mL)	5mL No anticoagulant (gold)	BD Vacutainer 367954	Stand for 30 min at room temperature before centrifuging	15 min x 1300 g @ 4˚C	Separate into 2 x 0.5mL aliquots	25-OH-D2 and 25-OH-D3	Freeze all aliquots and store at -80°	Keep vitamin D samples – send all together to HFL Sport Science at the end of the study.

All analysis done at King's College Hospital (KCH), apart from vitamin D for screening and all study days. BD (Becton-Dickinson, Oxford Science Park, OX4 4DQ). FBC, full blood count; LFT, liver function test; PTH, parathyroid hormone. 25-OH-D2 and 25-OH-D3 measured at HFL Sport Science. ‡2 mL Eppendorf tubes used.

APPENDIX 7 DRISK study blood handling protocol at screening and on study days

VISIT	TUBES		ICE/RT	CENTRIFUGE	SEPARATION‡	ANALYTES	STORAGE	TRANSPORT
Screening (9mL)	2 mL Fluoride oxalate (grey)	BD Vacutainer 368920	Room temperature	15 min x 1300 g @ 4˚C	Separate plasma into 2 x 0.4 mL aliquots	Glucose	Freeze 1 aliquot at -80° and send the other fresh to KCH	Same day to KCH at room temperature
	2 mL EDTA (lavender)	BD Vacutainer 367836	Room temperature	No	None	FBC	Room temperature	Same day to KCH at room temperature
	5 mL No anticoagulant (gold)	BD Vacutainer 367954	Stand for 30 min at room temperature before centrifuging	15 min x 1300 g @ 4˚C	Separate into 2 x 1 mL aliquots	LFT, FLIP and 25-OH-D	Freeze one 1mL aliquot and send the other 1mL fresh to KCH.	Same day to KCH at room temperature
Baseline, 6 wk and 12 wk visits (18 mL per visit)	4 mL EDTA (lavender)	BD Vacutainer 367839	Room temperature	15 min x 1300 g @ room temperature	Separate plasma into 2 x 0.5 mL aliquots and 1 x 0.6 mL within 2h	PTH and renin	Freeze both aliquots (within 2 h). Store both at -80°C.	Store and transport later in batches to KCH in dry ice.
	8.5 mL No anticoagulant (gold)	BD Vacutainer 367958	Stand for 30 min at room temperature before centrifuging	15 min x 1300 g @ room temperature	Separate into 3 x 0.5 mL and 2 x 1.0 mL aliquots	1st aliquot – Calcium, FLIP and hsCRP. 2nd aliquot – MMP-9 and C-peptide. 3rd aliquot – 25-OH-D2 and 25-OH-D3. 4th and 5th 1.0 mL aliquots – spares.	Freeze all aliquots and store at -80°	Keep vitamin D samples – these will be sent all together to HFL Sport Science at the end of the study in dry ice. Store and transport other samples later in batches to KCH.
	4.5mL Sodium Citrate (blue)	BD Vacutainer 367691	Room temperature	15 min x 1300 g @ room temperature	Separate into 2 x 1.0 mL aliquots	Fibrinogen and $FVII_c$	Freeze both aliquots and store at -80°	Store and transport later in batches to KCH in dry ice.
	2 mL Fluoride oxalate (grey)	BD Vacutainer 368920	Room temperature	15 min x 1300 g @ room temperature	Separate plasma into 2 x 0.4 mL aliquots	Glucose	Freeze both aliquot at -80°	Store and transport later in batches to KCH in dry ice.

All analysis apart from 25-OH-D2, 25-OH-D3, fibrinogen and $FVII_c$ for visits 1, 2 and 3 done at King's College Hospital (KCH). 25-OH-D2 and 25-OH-D3 measured at HFL Sport Science. BD (Becton-Dickinson, Oxford Science Park, OX4 4DQ). FBC; full blood count; LFT, liver function test; PTH, parathyroid hormone; FLIP, full lipid profile; MMP-9, matrix metalloproteinase-9; hsCRP, high sensitivity C-reactive protein; ‡2 mL Eppendorf tubes used.

APPENDIX 8 DEQAS Proficiency Certificate for the Department of Clinical Biochemistry at King's College Hospital (KingsPath)

APPENDIX 9 Lifestyle questionnaire used in DRISK, CRESSIDA and MARINA studies. Pages 2-10 used for D_2/D_3 comparison study.

CONFIDENTIAL

DRISK Study

Date: _____

First Name: _____

Surname: _____

This questionnaire asks for some background information about you, especially about what you eat. Please fill it in at home and bring it with you to the clinic. Please answer every question. If you are uncertain about how to answer a question then do the best you can, but please do not leave a question blank. If you have any problems with the questions please ask the researcher to help when you come for your appointment.

Your answers will be treated as strictly confidential and will be used only for medical research.

PARTICIPANT'S LIFESTYLE AND HEALTH QUESTIONNAIRE

Please complete this section before going to question 1.

Date of birth: ☐ day ☐ month ☐ year

Are you male or female? Male ☐ Female ☐

How tall are you? ☐ feet and ☐ inches **or** ☐ centimetres

How much do you weigh? ☐ stones and ☐ pounds **or** ☐ kilogrammes

How old were you when you left school? ☐ years old

Do you eat any meat (including bacon, ham, poultry, game, meat pies, sausages)? Yes ☐ No ☐

If no, how old were you when you last ate meat? ☐ years old

Do you eat any fish? Yes ☐ No ☐

If no, how old were you when you last ate fish? ☐ years old

Do you eat any dairy products (including milk, cheese, butter, yogurt)? Yes ☐ No ☐

If no, how old were you when you last ate dairy products? ☐ years old

Do you eat any eggs (including eggs in cakes and other baked foods)? Yes ☐ No ☐

If no, how old were you when you last ate eggs? ☐ years old

Listed below are 130 food items divided into sections according to food type. For each food there is an amount shown, either a "medium serving" or a common household unit such as a slice or teaspoon. Please put a tick (✓) in the box to indicate how often, **on average**, you have eaten the specified amount of each food **during the last 12 months**.

EXAMPLES:

For white bread the amount is one slice, so if you ate 4 or 5 slices a day, you should put a tick in the column headed "4-5 per day".

FOODS AND AMOUNTS	AVERAGE USE IN LAST 12 MONTHS								
BREAD AND SAVOURY BISCUITS (one slice or biscuit)	Never or less than once/month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
White bread and rolls								✓	

For chips, the amount is a "medium serving", so if you had a helping of chips twice a week you should put a tick in the column headed "2-4 per week".

FOODS AND AMOUNTS	AVERAGE USE IN LAST 12 MONTHS								
POTATOES, RICE AND PASTA (medium serving)	Never or less than once/month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Chips				✓					

For very seasonal fruits such as strawberries and raspberries you should estimate your average use when the fruits are in season, so if you ate strawberries or raspberries about once a week when they were irries, kiwi fruit ✓

1. Please estimate your average food use as best you can, and please answer every question.

MEAT AND FISH

Did you eat any meat or fish in the last 12 months? Yes ☐ No ☐

If no, please go to next page

If yes , please fill in this page

PLEASE PUT A TICK (✓) ON EVERY LINE

FOODS AND AMOUNTS	AVERAGE USE IN LAST 12 MONTHS								
MEAT AND FISH (medium serving)	Never or less than once/month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Beef: roast, steak, mince, stew or casserole									
Beefburgers									
Pork: roast, chops, stew or slices									
Lamb: roast, chops or stew									
Chicken or other poultry e.g. turkey									
Bacon									
Ham									
Corned beef, Spam, luncheon meats									
Sausages									
Savoury pies, e.g. meat pie, pork pie, pasties, steak & kidney pie, sausage rolls									
Liver, liver paté, liver sausage									
Fried fish in batter, as in fish and chips									
Fish fingers, fish cakes									
Other white fish, fresh or frozen, e.g. cod, haddock, plaice, sole, halibut									
Oily fish, fresh or canned, e.g. mackerel, kippers, tuna, salmon, sardines, herring									
Shellfish, e.g. crab, prawns, mussels									
Fish roe, taramasalata									
	Never or less than once/month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day

What did you do with the visible fat on your meat?

Ate most of the fat ☐ Ate as little as possible ☐

Ate some of the fat ☐ Did not eat meat ☐

How often did you eat grilled or roast meat? ☐☐ times a week

How well cooked did you usually have grilled or roast meat?

Well done /dark brown ☐ Lightly cooked/rare ☐

Medium ☐ Did not eat meat ☐

PLEASE PUT A TICK (✓) ON EVERY LINE

FOODS AND AMOUNTS	AVERAGE USE IN LAST 12 MONTHS								
BREAD AND SAVOURY BISCUITS (one slice or biscuit)	Never or less than once/month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
White bread and rolls									
Brown bread and rolls									
Wholemeal bread and rolls									
Cream crackers, cheese biscuits									
Crispbread, e.g. Ryvita									
CEREALS (one bowl)									
Porridge, Readybrek									
Breakfast cereal such as cornflakes, muesli etc.									
POTATOES, RICE AND PASTA (medium serving)									
Boiled, mashed, instant or jacket potatoes									
Chips									
Roast potatoes									
Potato salad									
White rice									
Brown rice									
White or green pasta, e.g. spaghetti, macaroni, noodles									
Wholemeal pasta									
Lasagne, moussaka									
Pizza									
	Never or less than once/month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day

Please check that you have a tick (✓) on EVERY line

PLEASE PUT A TICK (✓) ON EVERY LINE

FOODS AND AMOUNTS	AVERAGE USE IN LAST 12 MONTHS								
DAIRY PRODUCTS AND FATS	Never or less than once/month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Single or sour cream (tablespoon)									
Double or clotted cream (tablespoon)									
Low fat yogurt, fromage frais (125g carton)									
Full fat or Greek yogurt (125g carton)									
Dairy desserts (125g carton)									
Cheese, e.g. Cheddar, Brie, Edam (medium serving)									
Cottage cheese, low fat soft cheese (medium serving)									
Eggs as boiled, fried, scrambled, etc. (one)									
Quiche (medium serving)									
Low calorie, low fat salad cream(tablespoon)									
Salad cream, mayonnaise (tablespoon)									
French dressing (tablespoon)									
Other salad dressing (tablespoon)									
The following on bread or vegetables									
Butter (teaspoon)									
Block margarine, wrapped, NOT tub, e.g. Stork,Krona (teaspoon)									
Polyunsaturated margarine, in tub, e.g. Flora, sunflower (teaspoon)									
Other soft margarine, dairy spreads, in tub, e.g. Blue Band, Clover (teaspoon)									
Low fat spread, in tub, e.g. Outline, Gold (teaspoon)									
Very low fat spread, in tub (teaspoon)									
	Never or less than once/month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day

Please check that you have a tick (✓) on EVERY line

PLEASE PUT A TICK (✓) ON EVERY LINE

FOODS AND AMOUNTS	AVERAGE USE IN LAST 12 MONTHS								
SWEETS AND SNACKS (medium serving)	Never or less than once/month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Sweet biscuits, chocolate , e.g. digestive (one)									
Sweet biscuits, plain, e.g. Nice, ginger (one)									
Cakes e.g. fruit, sponge, home baked									
Cakes e.g. fruit, sponge, ready made									
Buns, pastries e.g. scones, flapjacks, home baked									
Buns, pastries e.g. croissants, doughnuts, ready made									
Fruit pies, tarts, crumbles, home baked									
Fruit pies, tarts, crumbles, ready made									
Sponge puddings, home baked									
Sponge puddings, ready made									
Milk puddings, e.g. rice, custard, trifle									
Ice cream, choc ices									
Chocolates, single or squares									
Chocolate snack bars e.g. Mars, Crunchie									
Sweets, toffees, mints									
Sugar added to tea, coffee, cereal (teaspoon)									
Crisps or other packet snacks, e.g. Wotsits									
Peanuts or other nuts									
SOUPS, SAUCES, AND SPREADS									
Vegetable soups (bowl)									
Meat soups (bowl)									
Sauces, e.g. white sauce, cheese sauce, gravy (tablespoon)									
Tomato ketchup (tablespoon)									
Pickles, chutney (tablespoon)									
Marmite, Bovril (teaspoon)									
Jam, marmalade, honey (teaspoon)									
Peanut butter (teaspoon)									
	Never or less than once/month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day

Please check that you have a tick (✓) on EVERY line

PLEASE PUT A TICK (✓) ON EVERY LINE

FOODS AND AMOUNTS	AVERAGE USE IN LAST 12 MONTHS								
DRINKS	Never or less than once/month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Tea (cup)									
Coffee, instant or ground (cup)									
Coffee, decaffeinated (cup)									
Coffee whitener, e.g. Coffee-mate (teaspoon)									
Cocoa, hot chocolate (cup)									
Horlicks, Ovaltine (cup)									
Wine (glass)									
Beer, lager or cider (half pint)									
Port, sherry, vermouth, liqueurs (glass)									
Spirits, e.g. gin, brandy, whisky, vodka (single)									
Low calorie or diet fizzy soft drinks (glass)									
Fizzy soft drinks, e.g. Coca cola, lemonade (glass)									
Pure fruit juice (100%) e.g. orange, apple juice (glass)									
Fruit squash or cordial (glass)									
FRUIT (1 fruit or medium serving) **For very seasonal fruits such as strawberries, please estimate your average use when the fruit is in season**									
Apples									
Pears									
Oranges, satsumas, mandarins									
Grapefruit									
Bananas									
Grapes									
Melon									
Peaches, plums, apricots									
Strawberries, raspberries, kiwi fruit									
Tinned fruit									
Dried fruit, e.g. raisins, prunes									
	Never or less than once/month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day

Please check that you have a tick (✓) on EVERY line

PLEASE PUT A TICK (✓) ON EVERY LINE

FOODS AND AMOUNTS	AVERAGE USE IN LAST 12 MONTHS								
VEGETABLES Fresh, frozen or tinned (medium serving)	Never or less than once/month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Carrots									
Spinach									
Broccoli, spring greens, kale									
Brussels sprouts									
Cabbage									
Peas									
Green beans, broad beans, runner beans									
Marrow, courgettes									
Cauliflower									
Parsnips, turnips, swedes									
Leeks									
Onions									
Garlic									
Mushrooms									
Sweet peppers									
Beansprouts									
Green salad, lettuce, cucumber, celery									
Watercress									
Tomatoes									
Sweetcorn									
Beetroot									
Coleslaw									
Avocado									
Baked beans									
Dried lentils, beans, peas									
Tofu , soya meat, TVP, Vegeburger									
	Never or less than once/month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day

Please check that you have a tick (✓) on EVERY line

Your diet last year, continued

2. Are there any **other** foods which you ate more than once a week? Yes ☐ No ☐

If yes, please list below

Food	Usual serving size	Number of times eaten each week

3. What type of milk did you most often use?

Select one only

Full cream, silver ☐ Semi-skimmed, red/white ☐
Skimmed/fat free ☐ Channel Islands, gold ☐
Dried milk ☐ Soya ☐
Other ☐ specify ☐ None ☐

If you used soya milk, please describe brand and type ☐

4. How much milk did you drink each day, including milk with tea, coffee, cereals etc?

None ☐ Three quarters of a pint ☐
Quarter of a pint ☐ One pint ☐
Half a pint ☐ More than one pint ☐

5. Did you usually eat breakfast cereal, excluding porridge and Ready Brek mentioned earlier? Yes ☐ No ☐

If yes, which brand and type of breakfast cereal, including muesli, did you usually eat?

List the one or two types most often used

Brand	Type

6. What kind of fat did you most often use for frying, roasting, grilling etc?

Select one only

Butter ☐ Solid white vegetable fat ☐
Lard/dripping ☐ Margarine ☐
Vegetable oil ☐ None ☐

If you used vegetable oil, please give type e.g. corn, sunflower ☐

7. What kind of fat did you most often use for baking cakes etc?

Select one only

Butter ☐ Solid white vegetable fat ☐
Lard/dripping ☐ Margarine ☐
Vegetable oil ☐ None ☐

If you used margarine, please give type e.g. Flora, Stork ☐

8. How often did you eat food that was fried at home?

Daily ☐ Less than once a week ☐
4-6 times a week ☐ Never ☐
1-3 times a week ☐

9. How often did you eat fried food away from home?

Daily ☐ Less than once a week ☐
4-6 times a week ☐ Never ☐
1-3 times a week ☐

10. How often did you add salt to food while cooking?

Always ☐ Rarely ☐
Usually ☐ Never ☐
Sometimes ☐

11. How often did you add salt to any food at the table?

Always ☐ Rarely ☐
Usually ☐ Never ☐
Sometimes ☐

12. Did you regularly use a salt substitute (e.g. LoSalt)? Yes ☐ No ☐

If yes, which brand? _____

13. Have you regularly taken any vitamins, minerals, fish oils, fibre or other food supplements during the last 12 months? Yes ☐ No ☐

If yes, list brand and daily dose

Name and brand of supplements	Daily dose

14. In the last 12 months, have you eaten a modified diet for any of these reasons?

Tick more than one box if applicable

High blood pressure ☐ High blood cholesterol ☐
Stomach problems (e.g. ulcer or gastritis) ☐ Overweight/obesity ☐
Bowel problems (e.g. irritable bowel or diverticulitis) ☐ Diabetes ☐
Allergies (e.g. skin rash) ☐ Concern over eating a healthy diet ☐
Concern over a family history of illness ☐ Not modified my diet ☐
Other ☐ specify _____

15. When you were aged 20, about how many alcoholic drinks did you have each week?

Put "0" if none, "occ" if occasional but less than one drink a week

Please answer EACH line

Beer or cider	☐ pints each week
Wine	☐ glasses each week
Sherry or other fortified wine	☐ glasses each week
Spirits	☐ glasses (singles) each week

16. When you were aged 30, about how many alcoholic drinks did you have each week?

Put "0" if none, "occ" if occasional but less than one drink a week

Please answer EACH line

Beer or cider	☐ pints each week
Wine	☐ glasses each week
Sherry or other fortified wine	☐ glasses each week
Spirits	☐ glasses (singles) each week

Not yet aged 30 ☐

17. Have you ever smoked as much as one cigarette a day for as long as a year? Yes ☐ No ☐

If no, please go to question 18

If yes, how old were you when you started smoking cigarettes regularly? ☐☐ years old

Did you smoke at the following ages? If so, how many cigarettes did you smoke and were they usually filter cigarettes?

Age 20	☐☐☐ cigs per day	Filter ☐	No filter ☐	Non smoker ☐
Age 30	☐☐☐ cigs per day	Filter ☐	No filter ☐	Non smoker ☐
Age 40	☐☐☐ cigs per day	Filter ☐	No filter ☐	Non smoker ☐
Age 50	☐☐☐ cigs per day	Filter ☐	No filter ☐	Non smoker ☐

Do you smoke cigarettes now? Yes ☐ No ☐

If yes, how many cigarettes do you smoke each day? ☐☐☐ cigarettes

Do you usually smoke filter cigarettes? Yes ☐ No ☐

Do you usually smoke low tar cigarettes? Yes ☐ No ☐

Which brand do you normally smoke? ☐

How deeply do you inhale? Deeply into the lungs ☐ A little ☐ Not at all ☐

If you have stopped smoking, how old were you when you last smoked ? ☐☐ years old

18. Do you currently smoke cigars? Yes ☐ No ☐

19. Do you currently smoke a pipe? Yes ☐ No ☐

20. Approximately how much did you weigh when you were 20 years old?

☐☐ stones ☐☐ lbs **or** ☐☐☐.☐ kg

21. What is your present waist size? ☐☐ inches **or** ☐☐☐ centimetres

22. What is your present hip size? ☐☐ inches **or** ☐☐☐ centimetres

23. In a typical week during the last 12 months, how many hours did you spend on each of the following activities? **Put "0" if none**

Housework, such as cleaning, washing, cooking, child care		☐☐	hours per week
Do-it-yourself		☐☐	hours per week
Gardening	in summer	☐☐	hours per week
	in winter	☐☐	hours per week
Walking, including walking to work, shopping and leisure	in summer	☐☐	hours per week
	in winter	☐☐	hours per week
Cycling, including cycling to work and leisure	in summer	☐☐	hours per week
	in winter	☐☐	hours per week
Other physical exercise, such as keep-fit, aerobics, swimming, jogging, tennis	in summer	☐☐	hours per week
	in winter	☐☐	hours per week

24. In a typical week during the last 12 months, did you practise any of these activities vigorously enough to cause sweating or a faster heartbeat? Yes ☐ No ☐

If yes, for how many hours each week did you practise such vigorous physical activity?

☐☐ hours per week

25. In a typical day during the last 12 months, how many floors of stairs did you climb up?

Put "0" if none ☐☐ floors per day

26. Have you ever been told by a doctor that you have, or had, any of the following conditions?

Please tick all which apply and give the age at which each condition was first diagnosed.

Heart attack, coronary thrombosis, myocardial infarction	Yes ☐	at age ☐☐	yrs old	No ☐
Angina	Yes ☐	at age ☐☐	yrs old	No ☐
Stroke	Yes ☐	at age ☐☐	yrs old	No ☐
High blood pressure (hypertension)	Yes ☐	at age ☐☐	yrs old	No ☐
High blood cholesterol, hyperlipidaemia	Yes ☐	at age ☐☐	yrs old	No ☐
Diabetes	Yes ☐	at age ☐☐	yrs old	No ☐
Gallstones	Yes ☐	at age ☐☐	yrs old	No ☐
Polyps in the large intestine	Yes ☐	at age ☐☐	yrs old	No ☐
Cancer	Yes ☐	at age ☐☐	yrs old	No ☐

If yes, what type of cancer? ☐

Any other illnesses or operations?

.

	Age first diagnosed
☐	☐☐ yrs old
☐	☐☐ yrs old
☐	☐☐ yrs old
☐	☐☐ yrs old

27. Are you currently receiving long-term treatment for any illness or condition? Yes ☐ No ☐

If yes, please give details:

Illness or condition	Treatment	Dose	Frequency

28. Please give details of the ages of your mother and father, and whether they have ever had cancer or a heart attack. If you are adopted or if your parents remarried, please give details of your blood relatives only.

	Age now	OR	Age at death	Details of any cancer and/or heart attacks: Disease type	Age first diagnosed
Mother	☐☐ yrs		☐☐ yrs		☐☐ yrs
					☐☐ yrs
Father	☐☐ yrs		☐☐ yrs		☐☐ yrs
					☐☐ yrs

29. Do you have any brothers or sisters ? Yes ☐ No ☐

If yes, please give their ages, whether they are full or half brothers or sisters, and whether they have ever had cancer or a heart attack.

Brother OR Sister	Full OR Half	Age now	OR	Age at death	Details of any cancer and/or heart attacks: Disease type	Age first diagnosed
☐☐	☐☐	☐☐ yrs		☐☐ yrs		☐☐ yrs
						☐☐ yrs
☐☐	☐☐	☐☐ yrs		☐☐ yrs		☐☐ yrs
						☐☐ yrs
☐☐	☐☐	☐☐ yrs		☐☐ yrs		☐☐ yrs
						☐☐ yrs
☐☐	☐☐	☐☐ yrs		☐☐ yrs		☐☐ yrs
						☐☐ yrs
☐☐	☐☐	☐☐ yrs		☐☐ yrs		☐☐ yrs
						☐☐ yrs
☐☐	☐☐	☐☐ yrs		☐☐ yrs		☐☐ yrs
						☐☐ yrs
☐☐	☐☐	☐☐ yrs		☐☐ yrs		☐☐ yrs
						☐☐ yrs
☐☐	☐☐	☐☐ yrs		☐☐ yrs		☐☐ yrs
						☐☐ yrs
☐☐	☐☐	☐☐ yrs		☐☐ yrs		☐☐ yrs
						☐☐ yrs

30. How old were you when you finished full time education? ☐☐ years old

Not yet finished ☐

31. Do you have any of the following qualifications? **Tick all applicable**

CSE ☐ GCE "O" level ☐ "A" level, Highers ☐

Teaching diploma, HNC ☐ Degree ☐ None of these ☐

Other ☐ describe _____

32. Have you ever had a paid job ? Yes ☐ No ☐

If yes, please answer for your current or most recent job

What is/was your job title? _____

What do/did you do in your job? _____

What does/did the organization you work for make or do? _____

How many hours do/did you work each week? ☐☐ hours

Are/were you a Manager? ☐ Foreman/woman? ☐ Supervisor? ☐ None of these? ☐

Are/were you self-employed? Yes ☐ No ☐

In this job, which of the following best describes your physical activity. **Tick one only**

Sedentary occupation. You spend most of your time sitting (such as in an office). ☐

Standing occupation. You spend most of your time standing or walking. However, your work does not require intense physical effort (e.g. shop assistant, hairdresser, guard). ☐

Manual work. This involves some physical effort including handling of heavy objects and use of tools (e.g. plumber, electrician, carpenter). ☐

Heavy manual work. This involves very vigorous physical activity including handling very heavy objects (e.g. docker, miner, bricklayer, construction worker). ☐

Do you have a paid job at present ? Yes ☐ No ☐

If no, how would you describe yourself?

Housewife/husband ☐ Unemployed ☐

Retired ☐ Student ☐

Other ☐ describe _____

When did you last work? ☐☐☐☐ year Never ☐

33. What is your marital status?

Married or living as married ☐ Widowed ☐ Separated ☐
Divorced ☐ Single ☐

If you are not married or living as married, please go to question 34

If married or living as married, has your partner ever had a paid job? Yes ☐ No ☐

If yes, please answer for your partner's current or most recent job.

What is/was your partner's job title? _____

What does/did your partner do in this job? _____

What does/did the organization your partner works for make or do? _____

Is/was your partner a Manager? ☐ Foreman/woman? ☐ Supervisor? ☐ None of these? ☐

Is/was your partner self-employed? Yes ☐ No ☐

Does your partner have a paid job at present? Yes ☐ No ☐

If no, how would you describe your partner?

Housewife/husband ☐ Unemployed ☐ Retired ☐ Student ☐
Other ☐ describe _____

When did your partner last work? ☐☐☐☐ year Never ☐

34. To which of these groups do you consider you belong?

White ☐ Indian ☐ Pakistani ☐
Bangladeshi ☐ Chinese ☐ Black - Caribbean ☐
Black - other ☐ describe _____
Other ☐ describe _____

APPENDIX 10 Letter of favourable opinion for D_2/D_3 study from the South East London Research Ethics Committee

NHS
National Research Ethics Service
South East London REC 1
(Formerly Guy's REC)
Governor's Hall Suite
St Thomas' Hospital
London
SE1 7EH

Telephone: 020 7188 2260
Facsimile: 020 7188 2258

07 February 2011

Professor Tom Sanders
Head of the Nutritional Sciences Division
King's College London
Franklin-Wilkins Building
150 Stamford Street
London SE1 9NH

Dear Professor Sanders

Study Title: **Comparison of the bioavailability of ergocalciferol (vitamin D2) and cholecalciferol (vitamin D3) in healthy volunteers when consumed at different levels in a malted milk drink.**

REC reference number: **10/H0804/91**

Thank you for your letter of 01 February 2011, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

Ethical review of research sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

The favourable opinion applies to the following research site(s):

Research Site	Principal Investigator / Local Collaborator
King's College London	Professor Tom Sanders

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

For NHS research sites only, management permission for research ("R&D approval") should be obtained from the relevant care organisation(s) in accordance with NHS research governance arrangements. Guidance on applying for NHS permission for research is available in the Integrated Research Application System or at http://www.rdforum.nhs.uk.

Where the only involvement of the NHS organisation is as a Participant Identification Centre (PIC), management permission for research is not required but the R&D office should be notified of the study and agree to the organisation's involvement. Guidance on procedures for PICs is available in IRAS. Further advice should be sought from the R&D office where necessary.

Sponsors are not required to notify the Committee of approvals from host organisations.

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	*Version*	*Date*
Protocol	1	11 November 2010
Letter of invitation to participant	1	18 November 2010
Response to Request for Further Information		01 February 2011
REC application	Parts A - D	19 November 2010
REC application	SSI Form	14 December 2010
Student CV - Catherine Fisk		18 November 2010
Participant Information Sheet	2.0	05 January 2011
Interview Schedules/Topic Guides	1	18 November 2010
Questionnaire: Validated - Food Items		
Evidence of insurance or indemnity		01 August 2010
Referees or other scientific critique report	Letter from Anthony Young	18 November 2010
Letter from Statistician		17 November 2010
Referees or other scientific critique report	Letter from Sian Robinson	18 November 2010
Advertisement	Circular Email v:2.0	05 January 2011
Investigator CV	Professor Tom Sanders	16 November 2010
Participant Consent Form	2.0	05 January 2011
Covering Letter		19 November 2010
Summary/Synopsis	1	19 November 2010
Letter from Sponsor		19 November 2010
Screening Appointment Letter	1	18 November 2010
Not Eligible Letter	1	18 November 2010

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for

Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Now that you have completed the application process please visit the National Research Ethics Service website > After Review

You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the website.

The attached document *"After ethical review – guidance for researchers"* gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Progress and safety reports
- Notifying the end of the study

The NRES website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

We would also like to inform you that we consult regularly with stakeholders to improve our service. If you would like to join our Reference Group please email referencegroup@nres.npsa.nhs.uk.

10/H0804/91	**Please quote this number on all correspondence**

With the Committee's best wishes for the success of this project

Yours sincerely

Professor David Bartlett
Chair

Email: stephanie.hill@gstt.nhs.uk

Enclosures: "After ethical review – guidance for researchers" *SL- AR2 for other studies*

Copy to: *Keith Brennan, King's College London*

APPENDIX 11 Consent form for vitamin D_2/D_3 study

CONSENT FORM

KING'S College LONDON

University of London

Centre Name: King's College London

Study Number: 10/H0804/91

Patient Identification Number for this trial:

Title of Project: Comparison of the efficacy of vitamin D2 and D3 at raising vitamin D status.

Name of Researchers: Professor Tom Sanders and Miss Catherine Fisk

	Please initial box
1) The purpose and details of this study have been explained to me. I understand that this study is designed to further scientific knowledge and that all procedures have been approved by the South East London Research Ethics Committee 1.	☐
2) I confirm that I have read and understood this consent form and the information sheet for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily.	☐
3) I understand that I am under no obligation to take part in the study and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected. If I choose to withdraw from the study I will be asked whether the data collected from me can still be used. If I withdraw my consent from the study all of my samples and data will be disposed of.	☐

4) I confirm that I fit into the following criteria: ☐

- I am aged between 18 and 65
- I am not taking vitamin and mineral supplements (including cod-liver oil), or prescription calcium/vitamin D
- I have not had recent exposure to high UV-b light since 1 Dec 2010
- I do not have an intolerance to the study product (lactose, milk protein)
- I do not have chronic renal, liver or inflammatory bowel disease
- I do not have diabetes
- I am willing to restrict my consumption of oily fish to no more than 2 portions of oily fish per week

5) I agree to the collections and storage of my blood samples. ☐

6) I understand that all the information I provide will be treated in strict confidence ☐

7) I agree to participate in the above study. ☐

Your name .....

Your signature .....

Signature of researcher .....

Date .....

APPENDIX 12 Data from Public Health England in Chilton, near Oxford (the closest measurement station to London) showing that UV radiation was low during the study periods for the D_2/D_3 study (22/02/2011 – 13/04/2011) and the DRISK study (20/01/2012 - 03/05/2012 and 04/01/2013 - 30/04/2013)

Data used in this appendix is erythemally effective irradiance (UVReff). This is solar ultraviolet radiation (UVR) data that has been biologically weighted for the erythema (redness of the skin) action spectrum. As shown in **Figure A**, the action spectrum for human erythema and the action spectrum for the synthesis of pre-vitamin D are fairly similar in the UVB part of the solar spectrum (290-315 nm), and therefore this data is reasonable to use as a measure of UVB exposure with the potential to produce pre-vitamin D.

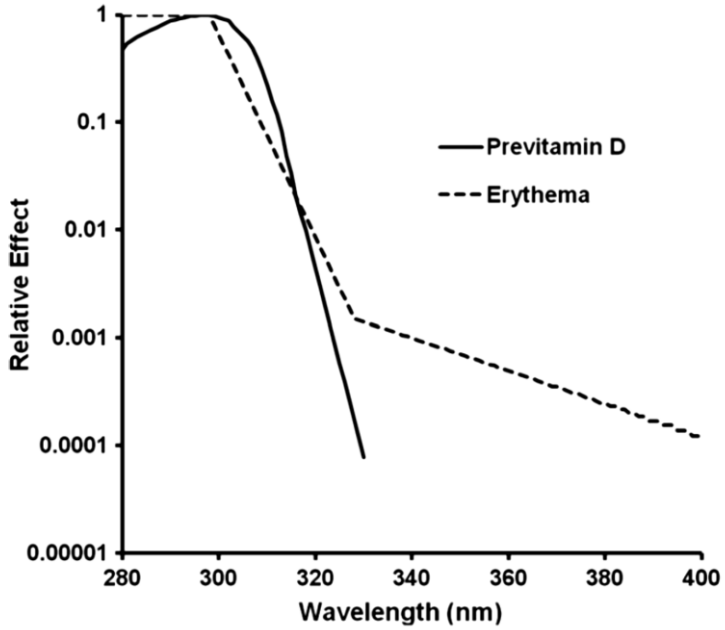

Figure A Commission Internationale de l'Éclairage (CIE) (1) action spectra for human erythema and the synthesis of previtamin D taken from (2).

Figure B demonstrates how UVReff changes over the course of a year with much higher values in the summer compared with the winter months (data shown are for 2011 when the D_2/D_3 comparison study was conducted). **Figure C** shows the changes in UVReff over each of the study periods. Values increased at the end of each study period, but remained low on average with means (SD) of 3.4×10^5 mW/m^2.s (5.2×10^5) for the D_2/D_3 comparison study and 6.1×10^5 mW/m^2.s (5.0×10^5) between 20/01/2012 and 03/05/2012 and 6.5×10^5 mW/m^2.s (6.9×10^5) between 04/01/2013 -

30/04/2013 for the DRISK study. Although UVReff values were quite high at the end of the 2013 study period, 25-OH-D_3 concentrations for participants taking part in the study during 2013 decreased overall from baseline to 12 wk from 33.9 to 31.8 nmol/L in the D_2 group and from 44.3 to 42.4 nmol/L in the placebo group.

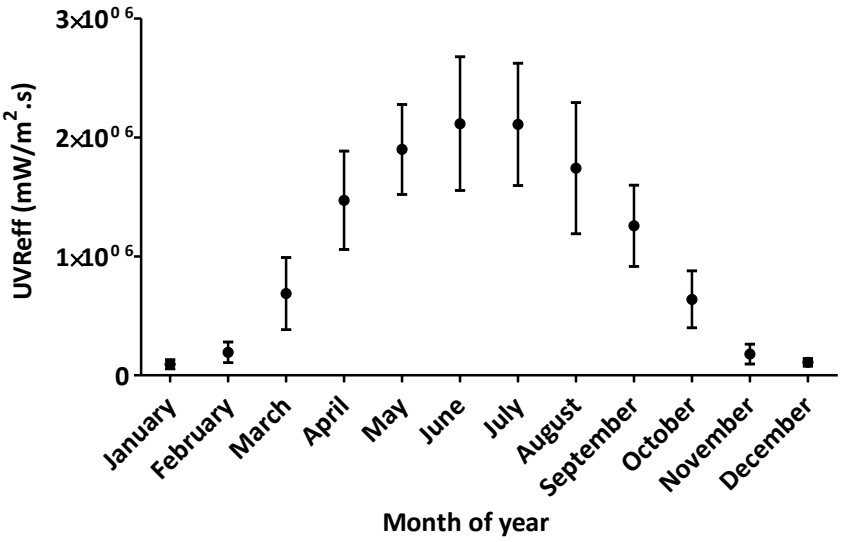

Figure B Mean (SD) erythemally effective irradiance (UVReff) in Chilton during 2011

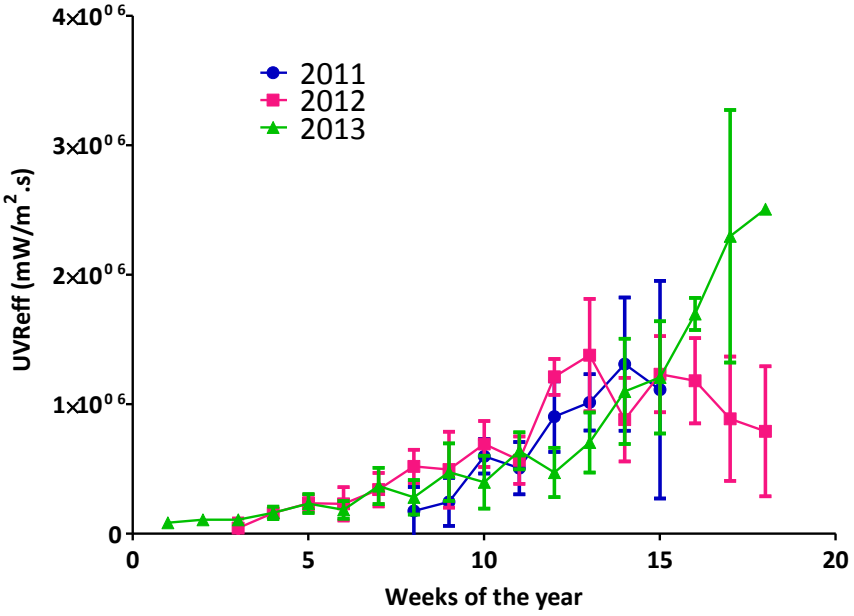

Figure C Mean (SD) erythemally effective irradiance (UVReff) in Chilton during the study periods for the D_2/D_3 comparison study (2011) and the DRISK study (2012 and 2013).

References

1. Bouillon, R., Eisman, J., Garabedian *et al.* (2006). Action spectrum for production of previtamin D3 in human skin. CIE Technical Report 174, Commission Internationale de l'Eclairage (CIE) Central Bureau, Vienna, Austria.

2. Springbett, P., Buglass, S. & Young, A.R. (2010). Photoreception and vitamin D status. *J Photochem Photobiol B.* **101**(2):160-8.

APPENDIX 13 Recruitment email for D_2/D_3 study

Circular e-mail for use for recruitment of volunteers for study ref 10/H0804/91, approved by the South East London Research Ethics Committee 1. This project contributes to the College's role in conducting research, and teaching research methods. You are under no obligation to reply to this email, however if you choose to, participation in this research is voluntary and you may withdraw at any time.

Title of Project: Comparison of the efficacy of vitamin D2 and D3 at raising vitamin D status.

There is a substantial body of evidence to suggest that there are likely to be health benefits of adequate vitamin D status in relation to a wide range of medical conditions such as osteomalacia, cardiovascular disease, muscle weakness, autoimmune diseases, and cancer. Levels of vitamin D insufficiency are high in the UK, particularly during the winter months. It is known that supplementation with vitamin D as either ergocalciferol (vitamin D2) or cholecalciferol (vitamin D3) can help improve vitamin D status. However, the evidence concerning the equivalence of these two forms in terms of their effectiveness at raising vitamin D levels is uncertain.

We require volunteers to help us investigate how effective different levels and types of vitamin D are at improving vitamin D status. If you are healthy, male or female, and are aged between 18 and 65 years, then you may be able to help us. You will be provided with an information sheet and given the opportunity to ask any questions you may have before proceeding. You will be asked to consume a malted milk drink every day for 4 weeks. You will be randomly allocated into one of 5 groups; malted milk drink not fortified with vitamin D (control), or malted milk drink fortified with 5mcg or 10mcg of either vitamin D2 or vitamin D3. We will ask you to come to the metabolic unit in the Franklin-Wilkins Building, which is at the Waterloo Campus of King's College London, on 6 occasions over a 5-6 week period. At each of these visits we will ask you to have a 2-3 tsp (10-15 mL) blood sample taken. This will be taken by a trained medical professional.

Exclusion factors are:

- Blood pressure >160/105 mm Hg
- Body Mass Index <18.5 and >35 kg/m2
- Anyone taking vitamin and mineral supplements (including cod-liver oil), or prescription calcium/vitamin D
- Recent exposure to high UV-b light since 1 Dec 2010
- Intolerance to study product (lactose, milk protein)
- Chronic renal, liver or inflammatory bowel disease
- Diabetes
- Unwilling to restrict consumption of oily fish to no more than 2 portions of oily fish per week.

This study will take place between February and April 2011 and you will be paid £50 on completion of the study as compensation for time spent on the study. You will also be reimbursed for travel expenses. If you are interested or have any questions relating to the study please contact Catherine Fisk at catherine.fisk@kcl.ac.uk or on 020 7848 4301.

APPENDIX 14 Participant information sheet for vitamin D_2/D_3 study

KING'S College LONDON

University of London

INFORMATION SHEET FOR PARTICIPANTS

REC Protocol Number: 10/H0804/91

YOU WILL BE GIVEN A COPY OF THIS INFORMATION SHEET

Title of Project: Comparison of the efficacy of vitamin D2 and D3 at raising vitamin D status.

We would like to invite you to participate in this postgraduate research project. You should only participate if you want to; choosing not to take part will not disadvantage you in any way. Before you decide whether you want to take part, it is important for you to understand why the research is being done and what your participation will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information.

What is the purpose of the study?

There is a substantial body of evidence to suggest that there are likely to be health benefits of adequate vitamin D status in relation to a wide range of medical conditions such as osteomalacia, cardiovascular disease, muscle weakness, autoimmune diseases, and cancer. Levels of vitamin D insufficiency are high in the UK, particularly during the winter months. It is known that supplementation with vitamin D as either ergocalciferol (vitamin D2) or cholecalciferol (vitamin D3) can help improve vitamin D status. However, the evidence concerning the equivalence of these two forms in terms of their effectiveness at raising vitamin D levels is uncertain. The results of this project will be used to help design a longer term study looking at the effects of vitamin D on cardiovascular risk factors.

Study design and procedures

All participants will be asked to consume a malted milk drink every day for 4 weeks. They will be randomly allocated into one of 5 groups; malted milk drink not fortified with vitamin D (control), or malted milk drink fortified with 5µg or 10µg of either vitamin D2 or vitamin D3. Participants will be required to have a blood sample taken in the metabolic unit in the Franklin-Wilkins Building on up to 6 occasions (2-3 tsps) over a five week period.

Why have I been invited?

This study is a multi-centre study, which will take place in the metabolic unit facilities at King's College London (KCL). A total of 40 volunteers will be recruited. You have been chosen to participate in this study as we require healthy volunteers.

In order to participate in this study you need to be able to say 'Yes' to the following:

- I am aged between 18 and 65 years.
- I am not taking any vitamin and mineral supplements (including cod-liver oil), or prescription Calcium/vitamin D.
- I have not had exposure to high UV-b light since 1 Dec 2010.
- I do not have a known intolerance to the study product (lactose, milk protein).
- I do not have chronic renal, liver or inflammatory bowel disease.
- I do not have diabetes
- I do not eat more than 2 portions of oily fish per week.

Do I have to take part?

It is up to you to decide whether to take part or not. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. You can withdraw from the study at any time by informing one of the researchers, and you do not have to give a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive. If you choose to withdraw from the study we will ask you whether we can use the data collected so far from you. If you withdraw your consent from the study we will dispose of all of your samples and data.

What will happen to me if I take part and what will I have to do?

If you would like to participate you first need to complete a screening interview with us over the telephone, (approx. 5 minutes), after which potentially eligible volunteers will be invited to attend a clinic screening appointment (approx. 25 minutes) in the Metabolic Unit, 4th Floor, Corridor A, Franklin-Wilkins Building, KCL.

The study will be explained in detail and you will be able to ask any questions to ensure you will be giving fully informed consent. Following the signing of the consent form, your height, weight and blood pressure will be measured, and a blood sample (14 mls, 2-3 tsps) will be taken.

If you are eligible to take part and you wish to participate, you will then be asked to attend the Metabolic Unit on five further occasions for study visits. These visits will take around 10-15 minutes each. The first visit will be at baseline (approximately a week after screening), and each further visit will occur every week for the next four weeks so that we are able to monitor changes in your vitamin D status. We shall ask you to avoid eating more than two portions of oily fish per week during the study. At each visit you will be asked to report to the Metabolic Unit at a pre-arranged time and we will take a small blood sample (10 ml, 2 teaspoons).

You will be compensated £50 for your time upon completion of the study. You will also be compensated for reasonable travel costs.

If you decide to take part, please let us know if you have been involved in any other study in the last year.

What will happen to the blood samples that I give?

All of the blood samples that we collect from you will be analysed to determine your vitamin D status. Liver function and haematology tests will be performed on your screening blood sample. The screening, baseline and 4 week visit blood samples will be analysed for calcium and parathyroid hormone. Analysis will take place under the supervision of investigators at KCL. All samples will be anonymised, and in the unlikely event that samples are to be used in alternative research, informed consent will be re-requested.

What are the possible disadvantages, risks and benefits of taking part?

The risk associated with the work is minimal but blood collection does include a very small risk of bruising. The potential health benefits of participation in this study include having the liver function and haematology tests, and determination of your vitamin D status, serum calcium concentrations and parathyroid hormone concentrations. You will be provided with copies of your screening results and results from the intervention. If any of your results are found to be abnormal we will discuss these with you and ask your permission to contact your GP.

What if there is a problem?

If this study has harmed you in any way you can contact KCL using the details below for further advice and information: Miss Catherine Fisk, catherine.fisk@kcl.ac.uk (telephone

020 7848 4301), Diabetes and Nutritional Sciences Division, King's College London, Franklin-Wilkins Building, 150 Stamford Street, London, SE1 9NH.

Will taking part in the study be kept confidential?

Subject confidentiality and anonymity will be observed throughout the study by use of subject codes in place of names, and the storage of subject details in a secure place. Only the investigators at KCL will have access to this data. Representatives of GlaxoSmithKline Consumer Healthcare and/or appropriate Independent Ethics Committee representatives and regulatory authorities may also review medical records without violating your confidentiality.

Who is organising and funding this research?

This study has been organised by Professor T Sanders (Head of Diabetes and Nutritional Sciences Division at King's College London), Miss Catherine Fisk (PhD Student at King's College London) and Dr. Hannah Theobald (Claims Innovator at GlaxoSmithKline). This is a GlaxoSmithKline sponsored study and is financially supported by GSK Consumer Healthcare.

Who has reviewed this study?

This study has been reviewed by the South East London Research Ethics Committee 1 to protect your safety, rights, wellbeing and dignity.

Thank you for your interest.
For further information, please contact:
Catherine Fisk
Division of Diabetes and Nutritional Sciences,
King's College London,
Room 4.46A Franklin Wilkins Building, 150 Stamford Street,
London SE1 9NH
Email: catherine.fisk@kcl.ac.uk
Tel: 020 7848 4301

Chief Investigator: Professor Tom Sanders Tel: 0207 848 4273

APPENDIX 15 Telephone interview for vitamin D_2/D_3 study

Telephone Interview

KING'S College LONDON
University of London

Participant details

Date .....

Name ..... M ☐ F ☐

Address .....

.....

Date of Birth .....

Age ..... **EXCLUDE If < 18 or > 65 Years**

Ethnicity .....

Phone Number: Day ..... Evening .....

Best time to phone .....

General Practitioner .....

Of (medical practice).....

.....

GP telephone number.....

Health

We would now like to ask you some Health questions. If there are any questions you would prefer not to answer please let us know.

Do you currently take any vitamin, mineral or oil supplements, including prescription vitamin D or calcium? If **Yes**, please give details. **Exclude if taking prescription vitamin D or calcium**	YES ☐	NO ☐
Are you prepared to stop taking any vitamin, mineral, or oil supplements for the duration of the study, i.e. 4 weeks in total? **Exclude if No**	YES ☐	NO ☐
Do you have any food allergies or intolerances? If **Yes**, please give details. **Exclude if intolerant to lactose or milk protein**	YES ☐	NO ☐
Are you prepared eat no more than 2 portions of oily fish (mackerel, salmon, etc) for the duration of the study, i.e. 4 weeks in total? **N.B.** White fish can still be eaten throughout **Exclude if No**	YES ☐	NO ☐
Have you been on holiday abroad to a lower latitude country, or used a tanning sunbed since 1 Dec 2010? **Exclude if Yes**	YES ☐	NO ☐
Do you have any plans to go on holiday to a lower latitude country, or use a tanning sunbed before the end of March 2011? **Exclude if Yes**	YES ☐	NO ☐

Do you know your Body Mass Index? BMI = ...

If not, please can you tell us your Weight..... ..... & Height .....

Exclude if BMI<18.5 and >35 kg/m^2 (to be confirmed at Screening Visit)

Do you have a history of any of the following conditions?

	YES	NO
(i) Chronic inflammatory bowel disease	☐	☐
(ii) Chronic renal disease	☐	☐
(iii) Chronic liver disease	☐	☐
(iv) Diabetes	☐	☐

If Yes to any, exclude from study

Suitability

Suitable? YES ☐ NO ☐

If yes, book Screening Visit

Date ..... Time .....

Investigator Signature .....
Date .....

Relevant information added to database ☐

Signature .....
Date .....

APPENDIX 16 Reference ranges for assays

Screening assays

Males

Plasma glucose:	Normal ≤ 6 mmol/L

Liver function test

Total protein:	60-80 g/L
Albumin:	35-50 g/L
Total Bilirubin:	3-20 µmol/L
Alkaline Phosphatase (ALP):	30-130 IU/L
Aspartate transaminase (AST):	10-50 IU/L
Gamma GT:	1-55 IU/L
Globulin:	25-35 g/L

Full blood count

White blood cells (WBC):	4.00-11.00 10ˆ9/L
Red blood cells (RBC):	4.5-5.8 10ˆ12/L
Haemoglobin(Hb):	13.0-16.5 g/dL
Packed cell volume (PCV):	0.400-0.540 L/L
Mean corpuscular volume (MCV):	77-95 fL
Mean corpuscular haemoglobin (MCH):	25-34 pg
Mean corpuscular haemoglobin concentration (MCHC):	33.0-37.0 g/dL
Red cell distribution width (RDW):	11.0-15.0 %
Platelet count (PLT):	150-450 10ˆ9/L
Mean platelet volume:	7.4-10.4 fL
Neutrophil count:	2.2-6.3 10ˆ9/L
Lymphocyte count:	1.3-4.0 10ˆ9/L
Monocyte count:	0.2-1.0 10ˆ9/L
Eosinophil count:	0-0.4 10ˆ9/L
Basophils:	0-0.1 10ˆ9/L

Females

Plasma glucose:	Normal ≤ 6 mmol/L

Liver function test

Total protein:	60-80 g/L
Albumin:	35-50 g/L
Total Bilirubin:	3-20 µmol/L
Alkaline Phosphatase (ALP):	45-130 IU/L

AST:	3-35 IU/L
Gamma GT:	1-55 IU/L
Globulin:	25-35 g/L

Full blood count

White blood cells:	4.00-11.00 10ˆ9/L
Red blood cells:	3.8-5.8 10ˆ12/L
Haemoglobin:	11.5-15.5 g/dL
PCV:	0.370-0.470 L/L
MCV:	77-95 fL
MCH:	25-34 pg
MCHC:	33.0-37.0 g/dL
RDW:	11.0-15.0 %
PLT:	150-450 10ˆ9/L
Mean platelet volume:	7.4-10.4 fL
Neutrophil count:	2.2-6.3 10ˆ9/L
Lymphocyte count:	1.3-4.0 10ˆ9/L
Monocyte count:	0.2-1.0 10ˆ9/L
Eosinophil count:	0-0.4 10ˆ9/L
Basophils:	0-0.1 10ˆ9/L

Study visit assays

Parathyroid hormone (PTH):	10-70 ng/L
Calcium corrected for albumin:	2.15-2.6 nmol/L
Direct renin :	5.4 – 60 mIU/L
High sensitivity C-reactive protein:	Low: less than 1.0 mg/L Average: 1.0 to 3.0 mg/L High: above 3.0 mg/L
Matrix metalloproteinase-9:	169 – 705 ng/mL
C-peptide:	298 – 2350 pmol/L
Fibrinogen:	1.5 – 4.5 g/L
$FVII_C$:	50 - 200% of standard
Total cholesterol:	ideally ≤5.0 mmol/L
HDL-cholesterol:	>1.0 mmol/L in men and >1.3 mmol/L in women
LDL-cholesterol:	<3.0 mmol/L
Triglycerides:	<1.7 mmol/L
TC:HDL-C	Ideal <3

APPENDIX 17 Malted milk drink analysis for D_2/D_3 study

Analysis of samples at the beginning of the study - International Food Network Ltd, Science & Technology Centre, Reading, RG6 6BZ

Treatment group	Metabolite measured	Result (µg/100g)	Average per 25g serving (µg)
Placebo	D_2	<0.5	<0.125
	D_2	<0.5	<0.125
	D_3	<0.5	<0.125
	D_3	<0.5	<0.125
Vit D_2 5µg	D_2	6.6	1.65
	D_2	7.2	1.80
Vit D_2 10µg	D_2	15.6	3.90
	D_2	16.4	4.10
Vit D_3 5µg	D_3	21.8	5.45
	D_3	19.8	4.95
Vit D_3 10µg	D_3	42.4	10.6
	D_3	37.4	9.35

Analysis of samples at the end of the study – LGC (Government Chemist), Queens Road,Teddington, Middlesex, TW11 0LY

Treatment group	Metabolite measured	Result (µg/100g)	Average per 25g serving (µg)
Placebo	D_2	Not detected	Not detected
	D_3	Not detected	Not detected
Vit D_2 5µg	D_2	19.05	4.76
Vit D_2 10µg	D_2	30.08	7.52
Vit D_3 5µg	D_3	11.49	2.87
Vit D_3 10µg	D_3	20.35	5.09

APPENDIX 18 Raw data for 25-OH-D_2, 25-OH-D_3 and total 25-OH-D measured by ultra high pressure liquid chromatography tandem mass spectrometry (UPLC-MS/MS) in study 1 (vitamin D_2/D_3 comparison).

25-OH-D_2 (nmol/L) by treatment group over 4 wk of supplementation with 5 or 10 µg D_2 or D_3/d, or placebo (a value of 2.4 nmol/L has been assigned to values below the level of detection of 2.423 nmol/L)

Subject ID	Treatment allocation	Screening	Baseline	Wk 1	Wk 2	Wk 3	Wk 4
3	Placebo	5.03	5.01	4.91	5.42	4.11	4.67
19	Placebo	0.00	0.00	0.00	0.00	0.00	0.00
20	Placebo	2.82	0.00	0.00	0.00	3.02	0.00
24	Placebo	5.28	5.06	4.65	4.18	3.84	2.92
27	Placebo	0.00	0.00	0.00	0.00	0.00	0.00
39	Placebo	0.00	0.00	0.00	0.00	0.00	0.00
41	Placebo	0.00	3.26	0.00	0.00	0.00	0.00
43	Placebo	4.16	3.96	3.87	4.62	4.55	5.30
2	D2 10µg	0.00	0.00	16.27	27.51	41.34	38.45
4	D2 10µg	2.42	2.42	10.17	16.00	19.07	21.79
5	D2 10µg	2.46	3.33	9.95	12.55	14.59	12.23
9	D2 10µg	2.70	3.26	10.97	14.32	18.05	21.11
25	D2 10µg	4.35	3.77	10.87	15.27	18.68	21.45
26	D2 10µg	2.42	2.53	4.28	4.13	4.40	4.99
35	D2 10µg	2.42	2.42	13.84	19.77	22.89	27.00
40	D2 10µg	2.42	2.42	5.64	10.94	15.18	16.25
1	D2 5µg	2.42	2.42	7.20	11.94	13.40	14.32
8	D2 5µg	2.42	2.42	8.32	10.63	13.96	13.81
12	D2 5µg	25.56	26.61	31.69	32.01	34.61	34.85
21	D2 5µg	4.55	3.26	6.83	8.22	10.53	10.34
22	D2 5µg	5.47	4.94	11.38	13.72	17.32	19.67
23	D2 5µg	2.42	2.42	5.23	10.09	11.70	11.33
33	D2 5µg	2.42	2.42	2.42	2.42	2.42	2.42
48	D2 5µg	2.70	2.42	8.12	11.65	12.70	14.52
11	D3 10µg	2.42	2.42	2.42	2.42	2.42	2.42
15	D3 10µg	2.42	2.42	2.42	2.42	2.42	2.42
16	D3 10µg	3.06	2.43	2.89	2.42	2.42	2.42
17	D3 10µg	2.42	2.42	2.42	2.42	2.42	2.42
30	D3 10µg	2.42	2.42	2.50	2.42	2.42	2.42
37	D3 10µg	2.42	2.42	2.42	2.85	2.42	2.42
44	D3 10µg	2.42	2.42	2.42	2.42	2.42	2.42
47	D3 10µg	2.42	2.42	2.42	2.42	2.42	2.42
6	D3 5µg	2.42	2.42	2.42	2.42	2.42	2.42
13	D3 5µg	2.42	2.42	2.42	2.63	2.42	2.42
28	D3 5µg	2.42	2.42	2.42	2.42	2.42	2.42
31	D3 5µg	2.42	2.42				
36	D3 5µg	2.42	2.42	2.42	2.42	2.42	2.42
38	D3 5µg	2.42	2.42	2.42	2.42	2.42	2.42
42	D3 5µg	2.42	2.42	2.42	2.42	2.42	2.42
46	D3 5µg	4.47	4.01	4.11	3.50	4.16	3.77

25-OH-D_3 (nmol/L) by treatment group over 4 wk of supplementation with 5 or 10 µg D_2 or D_3/d, or placebo

Subject ID	Treatment allocation	Screening	Baseline	Wk 1	Wk 2	Wk 3	Wk 4
3	Placebo	31.52	24.66	28.68	29.78	31.15	27.46
19	Placebo	55.81	40.76	36.62	33.45	35.74	36.39
20	Placebo	53.86	53.16	51.72	53.81	55.74	56.61
24	Placebo	14.18	14.73	12.36	11.01	10.28	9.81
27	Placebo	21.99	19.74	20.59	22.59	22.14	20.37
39	Placebo	30.15	26.86	25.41	25.16	24.36	21.72
41	Placebo	21.14	26.88	25.46	24.49	21.99	28.83
43	Placebo	43.26	37.56	38.21	31.82	33.55	31.77
2	D2 10µg	40.73	36.37	34.74	32.35	38.06	32.60
4	D2 10µg	29.48	25.58	27.76	30.45	27.03	22.86
5	D2 10µg	41.48	38.16	33.55	39.49	36.29	41.53
9	D2 10µg	63.65	56.88	56.46	49.37	46.35	49.25
25	D2 10µg	36.39	36.77	37.96	33.85	39.01	43.85
26	D2 10µg	51.94	48.00	48.60	55.36	47.82	53.89
35	D2 10µg	21.29	18.35	17.20	18.22	18.47	20.34
40	D2 10µg	62.72	52.57	56.14	45.65	51.32	42.68
1	D2 5µg	7.86	11.78	12.03	13.90	13.68	15.18
8	D2 5µg	75.60	72.23	65.32	62.28	61.20	60.90
12	D2 5µg	34.32	29.18	30.58	27.61	27.36	27.31
21	D2 5µg	65.79	52.67	44.73	40.31	44.05	41.23
22	D2 5µg	40.26	37.29	35.84	31.65	40.26	43.63
23	D2 5µg	53.24	50.77	50.67	51.44	47.35	51.87
33	D2 5µg	97.59	97.74	99.62	99.57	98.32	99.64
48	D2 5µg	35.12	28.40	27.83	30.45	26.88	25.51
11	D3 10µg	27.43	26.36	32.07	42.23	42.18	44.68
15	D3 10µg	15.10	13.40	23.66	34.05	34.92	36.69
16	D3 10µg	21.29	16.42	35.34	38.64	47.95	55.04
17	D3 10µg	17.27	18.55	26.76	36.54	38.76	41.98
30	D3 10µg	105.06	95.35	92.95	85.81	84.69	92.43
37	D3 10µg	28.60	25.38	33.85	40.16	44.68	46.08
44	D3 10µg	55.34	51.64	54.79	66.24	63.12	75.78
47	D3 10µg	20.89	19.97	32.82	35.19	42.58	44.45
6	D3 5µg	10.76	11.78	13.90	13.70	14.93	14.80
13	D3 5µg	81.57	69.89	70.56	70.81	62.95	69.81
28	D3 5µg	10.26	9.43	10.61	12.23	20.44	20.59
31	D3 5µg	12.08	11.81				
36	D3 5µg	31.92	32.20	35.47	39.54	42.76	47.87
38	D3 5µg	43.73	39.79	45.28	60.15	48.40	60.38
42	D3 5µg	40.78	35.39	39.01	45.75	50.62	58.46
46	D3 5µg	33.87	30.68	33.62	39.59	45.58	52.84

Total 25-OH-D (nmol/L) by treatment group over 4 wk of supplementation with 5 or 10 µg D_2 or D_3/d, or placebo

Subject ID	Treatment allocation	Screening	Baseline	Wk 1	Wk 2	Wk 3	Wk 4
3	Placebo	36.54	29.65	33.57	35.18	35.25	31.55
19	Placebo	58.23	43.18	39.04	35.87	38.17	38.82
20	Placebo	56.67	55.59	54.14	56.24	58.74	59.61
24	Placebo	19.44	19.77	16.98	15.18	14.11	13.64
27	Placebo	24.41	22.17	23.02	25.01	24.56	22.79
39	Placebo	32.58	29.28	27.83	27.58	26.78	24.14
41	Placebo	23.56	30.13	27.88	26.91	24.41	31.25
43	Placebo	47.40	41.51	42.07	36.43	38.08	36.31
2	D2 10µg	43.16	38.79	50.96	59.76	79.26	73.79
4	D2 10µg	31.90	28.01	37.89	46.40	46.03	41.86
5	D2 10µg	43.93	41.48	43.46	51.99	50.83	56.07
9	D2 10µg	66.34	60.13	67.39	63.64	64.33	67.23
25	D2 10µg	40.73	40.52	48.80	49.06	57.62	62.47
26	D2 10µg	54.37	50.52	52.86	59.48	52.21	58.28
35	D2 10µg	23.71	20.77	30.99	37.92	41.27	43.15
40	D2 10µg	65.15	54.99	61.76	56.56	66.44	57.80
1	D2 5µg	10.29	14.20	19.20	25.80	27.03	28.53
8	D2 5µg	78.03	74.66	73.61	72.87	75.11	74.81
12	D2 5µg	59.79	55.69	62.15	59.50	61.84	61.79
21	D2 5µg	70.33	55.91	51.54	48.50	54.55	51.73
22	D2 5µg	45.71	42.21	47.18	45.32	57.51	60.88
23	D2 5µg	55.66	53.19	55.88	61.50	59.01	63.52
33	D2 5µg	100.02	100.17	102.04	101.99	100.74	102.06
48	D2 5µg	37.81	30.83	35.92	42.06	39.53	38.16
11	D3 10µg	29.85	28.78	34.50	44.66	44.61	47.10
15	D3 10µg	17.52	15.83	26.09	36.47	37.34	39.11
16	D3 10µg	24.34	18.85	38.23	41.06	50.37	57.46
17	D3 10µg	19.70	20.97	29.18	38.96	41.19	44.41
30	D3 10µg	107.48	97.77	95.45	88.24	87.11	94.85
37	D3 10µg	31.03	27.81	36.27	43.00	47.10	48.50
44	D3 10µg	57.76	54.07	57.21	68.67	65.55	78.20
47	D3 10µg	23.31	22.39	35.25	37.62	45.01	46.88
6	D3 5µg	13.18	14.20	16.33	16.12	17.35	17.22
13	D3 5µg	83.99	72.31	72.99	73.43	65.37	72.24
28	D3 5µg	12.68	11.86	13.03	14.65	22.87	23.02
31	D3 5µg	14.50	14.23				
36	D3 5µg	34.35	34.62	37.89	42.37	45.18	50.30
38	D3 5µg	46.15	42.21	47.70	62.58	50.82	62.80
42	D3 5µg	43.21	37.82	41.44	48.18	53.04	60.88
46	D3 5µg	38.33	34.67	37.72	43.08	49.72	56.98

APPENDIX 19 Letter of favourable opinion for the DRISK study from the NRES Committee London-Westminster

NRES Committee London - Westminster
(Formerly St Thomas' Ethics Committee)
Ethics Committee Office
Governors' Hall Suite,
St Thomas' Hospital
London
SE1 7EH

Telephone: 020 7188 2257
Facsimile: 020 7188 2258

06 December 2011

Professor Thomas Sanders
Professor of Nutrition & Dietetics
King's College London
Nutritional Sciences Division
Franklin-Wilkins Building
150 Stamford Street, London
SE1 9NH

Dear Professor Sanders

Study title: **The effect of low dose vitamin D2, provided in a fortified malted milk drink, on cardiovascular risk.**
REC reference: **11/LO/1626**

Thank you for your letter of 15 November 2011, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair's Panel.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation [as revised], subject to the conditions specified below.

Ethical review of research sites

NHS sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

Non-NHS sites

The Committee has not yet been notified of the outcome of any site-specific assessment (SSA) for the non-NHS research site(s) taking part in this study. The favourable opinion does not therefore apply to any non-NHS site at present. We will write to you again as soon as one Research Ethics Committee has notified the outcome of a SSA. In the meantime no study procedures should be initiated at non-NHS sites.

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

Management permission ("R&D approval") should be sought from all NHS organisations involved in the study in accordance with NHS research governance arrangements.

Guidance on applying for NHS permission for research is available in the Integrated Research Application System or at http://www.rdforum.nhs.uk.

Where a NHS organisation's role in the study is limited to identifying and referring potential participants to research sites ("participant identification centre"), guidance should be sought from the R&D office on the information it requires to give permission for this activity.

For non-NHS sites, site management permission should be obtained in accordance with the procedures of the relevant host organisation.

Sponsors are not required to notify the Committee of approvals from host organisations

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	*Version*	*Date*
Advertisement - Poster	2	11 November 2011
Investigator CV		15 September 2011
Letter from Statistician		16 September 2011
Letter of invitation to participant	1.0	05 September 2011
Other: Recruitment email	2.0	11 November 2011
Other: Advertisement – newspaper	1.0	13 September 2011
Other: telephone interview	2.0	11 November 2011
Other: Ambulatory blood pressure monitor instructions (left arm)	1	13 September 2011
Other: ambulatory blood pressure monitor instructions (right arm)	1.0	13 September 2011
Other: Initial response letter	2.0	11 November 2011
Other: Evidence of sponsor insurance		01 August 2011
Other: Letter to patient - not eligible	2	11 November 2011
Participant Consent Form	2	11 November 2011
Participant Information Sheet	2.0	11 September 2011
Protocol	1	01 September 2011
Questionnaire: Validated -lifestyle and health		
Questionnaire: Validated-physical activity	1	15 September 2011
Questionnaire: Telephone	2	11 November 2011
REC application		23 September 2011
Referees or other scientific critique report		22 September 2011
Sample Diary/Patient Card	1	13 September 2011
Summary/Synopsis	1	05 September 2011

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Reporting requirements

The attached document *"After ethical review – guidance for researchers"* gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Notification of serious breaches of the protocol
- Progress and safety reports
- Notifying the end of the study

The NRES website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

Feedback

You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the website.

Further information is available at National Research Ethics Service website > After Review

11/LO/1626	**Please quote this number on all correspondence**

With the Committee's best wishes for the success of this project

Yours sincerely

S. Hirsch

pp

Dr Alan Ruben
Chair

Email: stella.hirsch@gstt.nhs.uk

Enclosures: "After ethical review – guidance for researchers" *[SL-AR2]*

Copy to: *Mr Keith Brennan*
Karen Ignatian, Guy's & St Thomas' Hospital

APPENDIX 20 R & D approval letter for DRISK study

Guy's and St Thomas' NHS
NHS Foundation Trust

Research & Development
16th Floor Tower Wing
Guy's Hospital
Great Maze Pond
London SE1 9RT
Tel: 020 7188 7188

Professor Philip Chowienczyk
Department of Clinical Pharmacology
CRF, 4th Floor
North Wing
St Thomas' Hospital
Westminster Bridge Road
London SE1 7EH

14th December 2011

Dear Professor Chowienczyk

Title: The effect of low dose vitamin D2, provided in a fortified malted milk drink, on cardiovascular risk.

In accordance with the Department of Health's Research Governance Framework for Health and Social Care, all research projects taking place within the Trust must receive a favourable opinion from an ethics committee and approval from the Department of Research and Development (R&D) prior to commencement.

- **Ethics Number:** 11/LO/1626
- **Sponsor:** KCL
- **Funder:** BBSRC
- **End Date:** 15/10/2013
- **Protocol:** Version 1
- **Site:** GSTFT
- **R&D Approval Date:** 14/12/2011
- **Chief Investigator:** Professor Thomas Andrew Bruce Sanders

NHS permission for the above research has been granted on the basis described in the application form, protocol and supporting documentation as listed in the ethics letter of favourable opinion letter dated 06/12/2011. I am pleased to inform you that we are approving the work to proceed within Guy's and St Thomas' NHS Foundation Trust and that the study has been allocated the Trust R&D registration number RJ111/N332. Please quote the R&D registration number in any communications with the R&D Department regarding your project.

Conditions of Approval:

- The principal investigator must ensure that the recruitment figures are reported.
- The principal investigator must notify R&D of the actual end date of the project.
- R&D must be notified of any changes to the protocol prior to implementation.
- The project must follow the agreed protocol and be conducted in accordance with all Trust Policies and Procedures especially those relating to research and data management.
- Members of the research team must have appropriate substantive or honorary contracts with the Trust prior to the study commencing. Any additional researchers who join the study at a later stage must also hold a suitable contract.
- **Storing tissue at the end of the study:** If tissues will be stored pending ethical approval for use in another project, then at the end of this study REC approval for the additional study must be in place, or the tissue has to be stored in HTA licensed premises.

Data Protection:
Please ensure that you are aware of your responsibilities in relation to The Data Protection Act 1998, NHS Confidentiality Code of Practice, NHS Caldicott Report and Caldicott Guardians, the Human Tissue Act 2004, Good Clinical Practice, the NHS Research Governance Framework for Health and Social Care, Second Edition April 2005 and any further legislation released during the time of this study.

The Principal Investigator is responsible for ensuring that Data Protection procedures are observed throughout the course of the project.

If the project is a clinical trial under the European Union Clinical Trials Directive the following must also be complied with:

1. The EU Directive on Clinical Trials (Directive 2001/20/EC) and UK's implementation of the Directive: The Medicines for Human Use (Clinical Trials) Regulations 2004;
2. The EU Directive on Principles and Guidelines for Good Clinical Practice (EU Commission Directive 2005/28/EC); and UK's implementation of the Directive: The Medicines for Human Use (Clinical Trials) Amendment Regulations 2006;
3. If a clinical trials team has to keep a subject in a department "out of hours" for whatever reason, the Senior Nurse for the Hospital should be informed of their presence – as should the Resuscitation Team.

Amendments:
Please ensure that you submit a copy of any amendments made to this study to the R&D Department.

ISRCTN registration:
If appropriate it is recommended that you register with the Current Controlled Trials website http://isrctn.org/. Find out more about registering for an International Standard Randomised Controlled Trial Number (ISRCTN) as part of the Portfolio application process. Non-commercial studies with an interventional component that are eligible for NIHR CRN support can register for an ISRCTN for free via the Portfolio Database.

Annual Progress Report:
It is obligatory that an annual report is submitted by the Chief Investigator to the research ethics committee, and we ask that a copy is sent to the R&D Department. The yearly period commences from the date of receiving a favourable opinion from the ethics committee.

Please submit a copy of the progress report on the anniversary of the Ethics favourable opinion **(December)**

Should you require any further information please do not hesitate to contact us.

In line with the Research Governance Framework, your project may be randomly selected for monitoring for compliance against the standards set out in the Framework. For information, the Trust's process for the monitoring of projects and the associated guidance is available from the Trust's intranet or on request from the R&D Department. You will be notified by the R&D Department if and when your project has been selected as part of the monitoring process. No action is needed until that time.

Thank you for registering your research project.

Yours sincerely

Maria Briana
R&D Governance Coordinator

APPENDIX 21 Consent form for DRISK study

CONSENT FORM

Centre Name: King's College London

Study Number: 11/LO/1626

Patient Identification Number for this trial:

Title of Project: The effect of low dose vitamin D2 supplementation, provided in a fortified malted milk drink, on cardiovascular risk.

Name of Researchers: Professor Tom Sanders, Miss Catherine Fisk, Professor Phil Chowienczyk and Dr Hannah Theobald.

	Please tick if you agree
1) The purpose and details of this study have been explained to me. I understand that this study is designed to further scientific knowledge and that all procedures have been approved by the NHS Research Ethics Committee London – Westminster.	☐
2) I confirm that I have read and understood this consent form and the information sheet for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily.	☐
3) I understand that I am under no obligation to take part in the study and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected. If I choose to withdraw from the study I will be asked whether the data collected from me can still be used. If I withdraw my consent from the study all of my samples and data will be disposed of.	☐

4) I understand that the study requests the following:

- I am not taking medication for lowering of blood cholesterol (statins) or blood pressure. ☐
- I do not have type 1 or type 2 diabetes mellitus. ☐
- I do not have chronic renal, liver or inflammatory bowel disease. ☐
- I do not smoke tobacco. ☐
- I have not had prolonged exposure to strong sunlight since Nov 2011. ☐
- I do not go to a lower latitude country, or use a tanning sunbed during the study period. ☐
- I do not have an intolerance to the study product (lactose, milk protein). ☐
- I am not taking vitamin and mineral supplements (including cod-liver oil), or prescription calcium/vitamin D. ☐
- I need to limit my consumption of oily fish to no more than 2 portions per week. ☐
- I need to avoid consuming soya milk throughout the study. ☐

5) I give permission for my GP to be notified that I am taking part in this study (you will still be able to take part if you do not want your GP to be informed). YES ☐ NO ☐

6) I agree to the collection and storage of my blood samples. ☐

7) I understand that all the information I provide will be treated in strict confidence. ☐

8) I agree to participate in the above study. ☐

Your name .....

Your signature .....

Signature of researcher .....

Date .....

APPENDIX 22 Recruitment email for DRISK study

"Circular email for use for recruitment of volunteers for study ref: 11/LO/1626, approved by the NHS Research Ethics Committee London - Westminster. This project contributes to the College's role in conducting research, and teaching research methods. You are under no obligation to reply to this email, however if you choose to, participation in this research is voluntary and you may withdraw at any time."

Title of project: The effect of low dose vitamin D2, provided in a malted milk drink, on cardiovascular risk.

We need volunteers to help us investigate whether vitamin D2 can influence factors associated with the development of cardiovascular disease, such as how well your blood vessels and circulation work (called 'vascular function').

If you are a healthy non-smoking male or non-smoking postmenopausal female and aged between 50 and 70 years then you may be able to help us.

What you will need to do:

- Complete a short telephone questionnaire (approximately 10 minutes).
- Attend a screening visit at either King's College London Franklin-Wilkins building, or the Clinical Research Facility at St Thomas' Hospital having fasted for 3 hours (nothing to eat or drink apart from water). Prior to this visit we will ask you to complete a lifestyle questionnaire that asks questions about your medical history and dietary habits (about 30 minutes to complete), and you will be asked to bring this along to the visit. We will measure your body mass index, height, weight and waist circumference and take a blood sample (20 mL/4 teaspoonfuls). We shall show you how to use an ambulatory blood pressure monitoring device, which you will wear for 24 hours. This may slightly restrict your activities (e.g. washing rather than having a shower) but normal daily functions can all be easily performed.
- If you are eligible for the study, after the screening visit you will need to come for three other visits (each approximately 2 hours in length) over the duration of the study (baseline, mid-point at 6 weeks, and final visit at 12 weeks). These will take place at the Clinical Research Facility at St Thomas' Hospital.
- At each of the visits you will be fitted with the ambulatory blood pressure monitor and asked to wear it for the next 24 hours. We will measure the health of your arteries and take a small blood sample (20 mL/4 teaspoonfuls). You will also be asked to complete a 20 minute test on a computer to see whether vitamin D supplementation can influence mood and the ability to conduct mental tasks.
- You would need to consume a malted milk drink, made up with hot water 3 times a week for 12 weeks.

For further information please contact us to request an information leaflet about the study. You will be compensated for your time on completion of the study. If you are interested or have any questions relating to the study please contact Catherine Fisk at drisk@kcl.ac.uk or on 020 7848 4301.

Appendix 23 Recruitment poster advertisement for DRISK study

KING'S College LONDON

University of London

VOLUNTEERS WANTED

FOR NUTRITION RESEARCH

We are conducting a study to find out whether vitamin D, provided in a malted milk drink, can influence factors associated with the development of heart disease when exposure to sunlight is low.

We are looking for non-smoking men and non-smoking postmenopausal women:

- Aged 50 – 70 years
- Willing to take part in a 3-month nutrition study
- Able to attend King's College London and St Thomas' Hospital, near Waterloo Station, for three visits spread throughout the study.

You will be reimbursed for your time and travel expenses and you will get a cardiovascular health check.

Please contact Catherine on 020 7848 4301 or email: drisk@kcl.ac.uk

APPENDIX 24 Newspaper advertisement in the London Metro for DRISK study

Vitamin D trial at King's College London

We need to find men and postmenopausal women to take part in a research study to find out if vitamin D, provided in a malted milk drink, can influence factors associated with the development of heart disease.

If you are in good health, aged 50-70 years, a non-smoker and interested in taking part, please email drisk@kcl.ac.uk for further information. You will be compensated for your time and travel expenses (we are near Waterloo station).

APPENDIX 25 Participant information sheet for DRISK study

INFORMATION SHEET FOR PARTICIPANTS

KING'S College LONDON

University of London

REC Protocol Number: 11/LO/1626

Title of Project: The effect of low dose vitamin D2, provided in a malted milk drink, on cardiovascular risk

You are being invited to take part in a research study. Before you decide, it is important you understand why the study is being done, and what it involves. Please take time to read this information carefully and discuss it with others if you wish. Please ask us if anything is not clear or you would like more details. Take time to decide if you wish to take part. Thank you for reading it.

What is the purpose of this study?

There is evidence to suggest that your vitamin D status may influence factors associated with the development of heart disease, such as how well your blood vessels and circulation work (called 'vascular function'). The aim of this study is to find out whether vitamin D2, provided in a malted milk drink, can influence these factors, and to investigate possible mechanisms by which this may occur.

Why have I been contacted?

You have been contacted as you have expressed an interest in our research. We are looking for non-smoking men or non-smoking postmenopausal women, aged 50-70 years, to take part in this study. If you would like to participate, you would complete a screening questionnaire with us over the telephone (approx. 10-15 min), after which you may be invited to a screening visit (approx. 30 min) for which you will need to have fasted for the previous 3 hours (nothing to eat or drink except water). This visit will take place at either the Franklin-Wilkins Building at King's College London (near Waterloo station), or at the Clinical Research Facility at St Thomas' Hospital. Before this visit, you would be sent a lifestyle questionnaire, which asks questions about your usual diet, use of medicines and medical history. You will be asked to return this at the screening visit. At the visit, you would have a blood sample taken (approx 20 ml or 4 teaspoons) and your height, weight and waist circumference measured so we can check whether you are suitable. Routine blood tests and vitamin D levels will be measured on the sample. We shall show you how to use an ambulatory blood pressure monitoring device, which you will wear for 24 hours. It will inflate intermittently and you will be given a specific instruction sheet for it. This may slightly restrict your activities (e.g. washing rather than having a shower) but normal daily functions can all be easily performed. We will recruit 40 volunteers in total.

Do I have to take part?

It is up to you to decide whether or not to take part. If you do decide to take part you will be asked to sign a consent form and will be given a copy to keep. However, you will still be free to withdraw from the study at any time and without giving a reason. This

will not affect any current or future medical care. If you choose to withdraw from the study we will ask you whether we can use the data collected so far from you. If you withdraw your consent from the study we will dispose of all of your samples and data.

What will happen to me if I take part?
If you decide to take part and are suitable for the study you will be allocated to one of two groups; one group will be asked to consume a malted milk drink with vitamin D added to it, and the other group will consume the drink with no vitamin D added to it. Both groups will be provided with powder sachets of the drink and asked to drink it made up with hot water 3 times a week for 12 weeks. There will be 24mcg vitamin D2 added to each of the vitamin D sachets. You will have an equal chance of being allocated to either of the groups, but you won't be able to choose which group you are in, or know which type of malted milk drink you are receiving. We will provide you with the drink sachets and the study will last for 12 weeks in total.

What do I have to do?
If you agree to take part and are eligible after the screening visit you will need to come for three other visits during the study (one baseline visit soon after the screening visit, one mid-point visit at 6 weeks, and one final visit at 12 weeks).

Each visit will take approximately 2 hours and will take place on a weekday morning at the Clinical Research Facility, St Thomas' Hospital. For each visit you will need to come in fasted, having had nothing to eat or drink except water after 10pm the previous evening. On the day before you will be asked to avoid strenuous physical activity and foods high in fat, caffeine and alcohol. At every visit we shall measure your weight, height and waist circumference and take a fasting blood sample (approx 20 ml or 4 teaspoons). At the end of each visit we shall fit you with the ambulatory blood pressure monitoring device, which you will wear for 24 hours. Vascular tests will be made at the three time-points to assess how stiff your arteries are by measuring how quickly the blood leaving your heart reaches an artery in your neck and an artery at the top of your leg. We do this by placing a sensor and cuff gently on your skin. After this we shall also make an ultrasound scan of an artery in your arm before and after inflating a blood pressure cuff for 5 mins. The cuff causes a 'tingling' sensation in your arm and may be uncomfortable, but does not cause pain.

At the baseline and mid-point visits you will be asked to complete a very short physical activity questionnaire. At the mid-point visit you will be asked to complete again the lifestyle questionnaire that you completed at screening. At both the baseline and final visit you will be asked to complete a 20 minute test on the computer to see whether vitamin D can influence mood and ability to conduct mental tasks.

At the end of the study you will receive a payment of £120 which we would advise you would be regarded as income for tax and benefits purposes. We will pay reasonable travel costs and request that you provide receipts where they are available.

What will happen to the blood samples that I give?
All of the blood samples that we collect from you will be anonymised, and in the unlikely event that samples are to be used in alternative research, informed consent will be re-requested. On completion of the study we will store anonymised data and destroy any stored blood samples.

Are there possible disadvantages and risks?
Taking blood samples may cause minor discomfort and there is a small chance of minor bruising. If a new diagnosis of type 2 diabetes or high blood pressure is made, this could affect your future insurance status (e.g. for life insurance or private medical insurance) but you will be aware of the diagnosis and able to seek treatment immediately.

What are the possible benefits of taking part?
You will be comprehensively screened for heart disease risk factors and diabetes. If we discover any of the results are out of the normal ranges (this does not necessarily mean that you are unwell), we will inform you and provide you with a letter and the results to give to your GP. Should any of the results be of clinical significance to your health, a consultant will provide a letter with recommendations for you to give to your GP. Although you will derive no further individual benefit, the knowledge gained from this study will help our research into the effects of vitamin D supplementation on risk of cardiovascular disease.

What will happen if anything goes wrong?
Any complaints you have about this study should be made to Professor Tom Sanders, King's College London, and will be fully investigated. If this study has harmed you in any way you can contact King's College London using the details on this leaflet for further advice and information.

Will my participation be kept confidential?
Subject confidentiality and anonymity will be observed throughout the study by use of subject codes in place of names, and the storage of subject details in a secure place. Only the investigators at KCL will have access to this data. Representatives of GlaxoSmithKline Consumer Healthcare and/or appropriate Independent Ethics Committee representatives and regulatory authorities may also review medical records without violating your confidentiality. With your consent, your GP will be informed that you are taking part in the study. However, if you do not wish your GP to be informed, you can still take part in the study. You will be notified if any results of significance to your health are found. We would advise you to inform any Private Medical Insurers that you are taking part in the study.

What will happen to the study results?
You will receive details of your own results once the study has been finished. This usually takes a long time, so you should not expect to receive your results for at least

3-4 months. We hope to publish the results of the whole study in a scientific journal. You will not be identified in any publication. We will be happy to discuss the overall results with you when the study is completed, and will let you know how you can get a copy of the published results if you wish.

Who is funding the study?
This is a GlaxoSmithKline sponsored study and is financially supported by GSK Consumer Healthcare.

Who has reviewed the study?
The NHS Research Ethics Committee London - Westminster.

And finally…
Thank you for taking time to read this information sheet and your interest in the study. If you decide to take part in the study, you will be given a copy of this information sheet and a signed consent form for you to keep.

For further information, please contact:

Catherine Fisk
Division of Diabetes and Nutritional Sciences,
King's College London,
Room 4.46A Franklin Wilkins Building,
150 Stamford Street,
London SE1 9NH
Email: drisk@kcl.ac.uk
Telephone: 020 7848 4301

Chief Investigator: Professor Tom Sanders, King's College London.

APPENDIX 26 Telephone questionnaire for DRISK study

Telephone Questionnaire – The effect of low dose vitamin D2 on cardiovascular risk

KING'S College LONDON
University of London

Participant details

Date .....

Name ..... M ☐ F ☐

Address .....

.....

Date of Birth .....

Age ..... **EXCLUDE If < 50 or > 70 Years**

Ethnicity .....

Phone Number: Day ..... Evening .....

Best time to phone .....

Email address .....

General Practitioner .....

Of (medical practice).....

.....

GP telephone number.....

Health

We would now like to ask you some Health questions. If there are any questions you would prefer not to answer please let us know.

Do you currently take any vitamin, mineral or oil supplements, including prescription vitamin D or calcium? If **Yes**, please give details. **Exclude if taking prescription vitamin D or calcium**	YES ☐	NO ☐
Are you prepared to stop taking any vitamin, mineral, or oil supplements for the duration of the study, i.e. 3 months in total? **Exclude if No**	YES ☐	NO ☐
Do you consume soya milk? **Exclude if Yes**	YES ☐	NO ☐
Do you have any food allergies or intolerances? If **Yes**, please give details. **Exclude if intolerant to lactose or milk protein**	YES ☐	NO ☐
Are you prepared eat no more than 2 portions of oily fish (mackerel, salmon, etc) per week for the duration of the study, i.e. 3 months in total? **N.B.** White fish can still be eaten throughout **Exclude if No**	YES ☐	NO ☐
Have you been on holiday abroad to a lower latitude country (e.g. Spain, Italy, Africa, Australia etc) or used a tanning sunbed since 1 Nov 2011? **Exclude if Yes**	YES ☐	NO ☐
Do you have any plans to go on holiday to a lower latitude country, or use a tanning sunbed before the end of April 2012? **Exclude if Yes**	YES ☐	NO ☐

Have you taken part in a clinical trial in the last 6 months? **Exclude if Yes**	YES ☐	NO ☐
Do you know your Body Mass Index? BMI = ... If not, please can you tell us your Weight..... & Height ..... **Exclude if BMI<18.5 and >35 kg/m^2 (to be confirmed at Screening Visit)**	YES ☐	NO ☐
Do you smoke? **Exclude if Yes**	YES ☐	NO ☐
Do you drink alcohol? If yes, how many units of alcohol would you consume in a typical week? (1 unit = 1 measure of sprits/ 1 small glass of wine/ 1 half pint of beer) ..... **Exclude if current intake of >21 Units/week for women, or >28 Units/week for men.**	YES ☐	NO ☐
(Women only) When was your last menstrual period?.....Years .....Months **Exclude if within the last year**		
Do you have a history of any of the following conditions?	YES	NO
(i) Heart attack (myocardial infarction) or stroke	☐	☐
(ii) Cardiovascular problems/angina	☐	☐
(iii) Stomach or inflammatory bowel disease	☐	☐
(iv) Kidney problems	☐	☐
(v) Liver disease, jaundice or anaemia	☐	☐
(vi) Diabetes **(exclude Type I and uncontrolled Type II)**	☐	☐
If Yes to any, exclude from study &/or take clinical advice.		
(vii) Chronic medical conditions	☐	☐
Please list any others and give details of recent hospital visits		
Are you currently taking any medications for the following conditions?	YES	NO
(i) Raised cholesterol e.g. fibrates or statins	☐	☐
(ii) Raised Blood Pressure e.g. diuretics, β-blockers, Ca-channel, ACE inhibitors, Angiotensin Receptor blockers	☐	☐
(iii) Immune system, e.g. antihistamines, anti-inflammatory	☐	☐
If yes to any, check if stable		

APPENDIX 27 Physical activity questionnaire for DRISK study

PHYSICAL ACTIVITY QUESTIONNAIRE

We are interested in finding out about the kinds of physical activities that people do as part of their everyday lives because it is associated with risk of cardiovascular disease. The questions are about the time you spent being physically active in the last 7 days. They include questions about activities you do at work, as part of your house and garden work, to get from place to place, and in your spare time for recreation, exercise or sport.
Your answers are important.

Please answer each question even if you do not consider yourself to be an active person.

THANK YOU FOR PARTICIPATING.

In answering the following questions,

- **vigorous** physical activities refer to activities that take hard physical effort and make you breathe much harder than normal.

- **moderate** activities refer to activities that take moderate physical effort and make you breathe somewhat harder than normal.

1a) During the last 7 days, on how many days did you do **vigorous** physical activities like heavy lifting, digging, aerobics, or fast bicycling?

Think about *only* those physical activities that you did for at least 10 minutes at a time.

_____ days per week ⇨

or

☐ **none**

1b. How much time in total did you usually spend on one of those days doing vigorous physical activities?

_____hours_____minutes

2a. Again, think *only* about those physical activities that you did for at least 10 minutes at a
time. During the last 7 days, on how many days did you do **moderate** physical activities like carrying light loads, bicycling at a regular pace, or doubles tennis? Do not include walking.

_____ days per week ⇨

or

☐ **none**

2b. How much time in total did you usually spend on one of those days doing moderate physical activities?

_____hours_____minutes

3a. During the last 7 days, on how many days did you **walk** for at least 10 minutes at a time? This includes walking at work and at home, walking to travel from place to place, and any other walking that you did solely for recreation, sport, exercise or leisure.

_____ days per week ⇨

or

☐ **none**

3b. How much time in total did you usually spend walking on one of those days?

_____hours_____minutes

The last question is about the time you spent sitting on weekdays while at work, at home, while doing course work and during leisure time. This includes time spent sitting at a desk, visiting friends, reading traveling on a bus or sitting or lying down to watch television.

4. During the last 7 days, how much time in total did you usually spend *sitting* on a **week day?**

____ hours _____ minutes

This is the end of questionnaire, thank you for participating

APPENDIX 28 Raw data for 25-OH-D_2, 25-OH-D_3 and total 25-OH-D in the DRISK study. Screening samples measured by chemiluminescent immunoassay and study day samples measured by ultra-high pressure liquid chromatography tandem mass spectrometry (UPLC-MS/MS).

25-OH-D_2 (nmol/L) by treatment group over 12 wk of supplementation with an intake equivalent to 10 µg D_2/d or placebo (a value of 2.4 nmol/L has been assigned to values below the level of detection of 2.423 nmol/L).

Subject ID	Treatment allocation	Baseline	Wk 6	Wk 12
4	Placebo	2.42	2.42	5.04
7	Placebo	4.97	5.84	6.18
9	Placebo	2.42	2.42	4.65
13	Placebo	2.42	2.42	2.42
14	Placebo	2.42	2.42	2.79
16	Placebo	2.42		
19	Placebo	2.42	6.01	10.23
20	Placebo	2.42	2.42	4.99
21	Placebo	2.42	2.42	2.42
27	Placebo	3.03	2.42	2.42
28	Placebo	2.54	2.42	2.42
33	Placebo	2.42	2.42	3.59
36	Placebo	3.39	3.15	2.42
38	Placebo	2.42	2.42	2.47
39	Placebo	2.42		
43	Placebo	2.42	2.42	2.42
44	Placebo	2.42	2.42	2.67
47	Placebo	2.42	2.42	2.42
48	Placebo	5.79	4.14	6.93
49	Placebo	6.11	4.36	2.69
1	D2	2.42	12.16	15.56
5	D2	2.42	18.88	23.38
8	D2	2.42	2.42	2.42
10	D2	2.42	16.36	28.28
11	D2	2.42	5.72	2.42
12	D2	2.42	12.62	18.10
18	D2	2.42	17.25	25.08
23	D2	2.42	16.60	23.50
25	D2	2.42	2.83	2.42
26	D2	2.42	34.65	42.89
29	D2	2.42	47.49	46.28
31	D2	2.42	30.05	27.38
32	D2	5.23	24.96	43.37
34	D2	2.42	34.41	27.38
35	D2	2.42	25.44	33.44
40	D2	4.39	16.28	22.99
41	D2	2.74	28.83	29.56
42	D2	2.42	32.47	36.35
45	D2	2.42	25.20	25.93

25-OH-D_3 (nmol/L) by treatment group over 12 wk of supplementation with an intake equivalent to 10 µg D_2/d or placebo

Subject ID	Treatment allocation	Baseline	Wk 6	Wk 12
4	Placebo	61.55	47.40	49.42
7	Placebo	43.01	32.30	32.32
9	Placebo	27.26	19.19	17.20
13	Placebo	38.31	28.88	26.63
14	Placebo	68.74	57.71	63.40
16	Placebo	51.64		
19	Placebo	18.92	14.45	20.67
20	Placebo	25.58	20.34	19.89
21	Placebo	34.74	36.84	38.21
27	Placebo	13.48	20.49	18.02
28	Placebo	24.94	18.57	16.45
33	Placebo	45.18	28.70	53.41
36	Placebo	16.55	19.17	20.29
38	Placebo	16.82	11.76	18.10
39	Placebo	52.92		
43	Placebo	40.44	47.67	58.16
44	Placebo	22.91	25.71	27.46
47	Placebo	64.65	90.60	72.63
48	Placebo	94.85	50.17	57.91
49	Placebo	94.35	98.84	81.12
1	D2	52.19	42.93	41.66
5	D2	63.97	44.23	43.78
8	D2	41.51	35.32	37.22
10	D2	46.43	28.85	28.60
11	D2	53.26	39.59	42.93
12	D2	17.57	18.84	23.74
18	D2	15.90	14.33	16.07
23	D2	65.35	51.47	50.69
25	D2	36.39	35.12	42.48
26	D2	23.89	22.41	29.95
29	D2	32.45	40.68	36.44
31	D2	37.94	29.95	33.45
32	D2	13.45	14.93	24.19
34	D2	35.44	41.68	31.95
35	D2	25.96	21.09	23.09
40	D2	29.45	26.46	24.14
41	D2	31.45	42.93	36.69
42	D2	50.92	36.19	32.95
45	D2	34.69	32.20	27.96

Total 25-OH-D (nmol/L) by treatment group over 12 wk of supplementation with an intake equivalent to 10 µg D_2/d or placebo

Subject ID	Treatment allocation	Screening	Baseline	Wk 6	Wk 12
4	Placebo	50.9	63.97	49.82	54.46
7	Placebo	32.7	47.97	38.14	38.50
9	Placebo	23.5	29.68	21.62	21.85
13	Placebo	31.9	40.74	31.30	29.06
14	Placebo	59.9	71.16	60.13	66.18
16	Placebo	37.9	54.07		
19	Placebo	21.5	21.34	20.46	30.89
20	Placebo	29.5	28.01	22.77	24.88
21	Placebo	31.9	37.17	39.26	40.64
27	Placebo	20.2	16.51	22.92	20.44
28	Placebo	28.5	27.48	20.99	18.87
33	Placebo	39.4	47.60	31.13	57.00
36	Placebo	24.5	19.94	22.32	22.72
38	Placebo	14.0	19.25	14.18	20.57
39	Placebo	49.2	55.34		
43	Placebo	36.2	42.86	50.10	60.58
44	Placebo	22.0	25.34	28.13	30.12
47	Placebo	46.2	67.07	93.03	75.06
48	Placebo	62.6	100.64	54.31	64.84
49	Placebo	50.7	100.45	103.20	83.81
1	D2	44.2	54.61	55.09	57.21
5	D2	53.4	66.40	63.10	67.16
8	D2	34.4	43.93	37.74	39.64
10	D2	48.7	48.85	45.21	56.88
11	D2	51.4	55.69	45.30	45.35
12	D2	18.2	19.99	31.47	41.84
18	D2	17.0	18.32	31.58	41.15
23	D2	54.4	67.77	68.07	74.20
25	D2	30.2	38.81	37.95	44.90
26	D2	32.4	26.31	57.06	72.84
29	D2	43.9	34.87	88.18	82.72
31	D2	47.7	40.36	60.00	60.83
32	D2	32.4	18.69	39.88	67.56
34	D2	40.7	37.87	76.09	59.33
35	D2	25.2	28.38	46.53	56.53
40	D2	46.9	33.84	42.74	47.13
41	D2	30.7	34.19	71.76	66.25
42	D2	40.4	53.34	68.66	69.29
45	D2	40.4	37.12	57.40	53.88

APPENDIX 29 24 h day-time and night-time blood pressure for DRISK study

Table A Effects of and intake equivalent to 10 µg vitamin D_2/d vs. placebo on day-time systolic (SBP) and diastolic (DBP) blood pressure and heart rate in healthy men and women aged 50-70 years.

	Placebo (*n* = 18)	Vitamin D_2 (*n* = 21)	Treatment effect (95% CIs)	*P* value
SBP, mm Hg				
Baseline 1	124 (11)	125 (13)		
Baseline 2	123 (14)	123 (10)		
Mean baseline	124 (12)	124 (11)		
6 wk	125 (12)	122 (12)		
12 wk	126 (12)	123 (9)		
Mean treatment	125 (11)	122 (10)		
Mean change (95% CIs)	1.6 (-1.0, 4.3)	-1.9 (-4.3, 0.6)		
Change adjusted	1.6 (-0.9, 4.0)	-1.8(-4.0, 0.5)	-3.3 (-6.7, 0.0)	*P*=0.050
DBP, mm Hg				
Baseline 1	76 (7)	76 (9)		
Baseline 2	76 (10)	75 (8)		
Mean baseline	76 (8)	76 (8)		
6 wk	78 (8)	75 (8)		
12 wk	77 (8)	75 (7)		
Mean treatment	77 (7)	75 (7)		
Mean change (95% CIs)	1.33 (-0.9, 3.6)	-1.0 (-3.0, 1.1)		
Change adjusted	1.2 (-0.9, 3.3)	-0.8 (-2.7, 1.1)	-2.1 (-4.9, 0.8)	*P*=0.155
Heart rate, beats/min				
Baseline 1	75 (6)	71 (8)		
Baseline 2	75 (7)	72 (9)		
Mean baseline	75 (6)	71 (8)		
6 wk	75 (6)	68 (8)		
12 wk	76 (7)	74 (8)		
Mean treatment	76 (6)	71 (7)		
Mean change (95% CIs)	0.6 (-1.3, 2.6)	-0.2 (-2.2, 1.6)		
Change adjusted	0.5 (-1.3, 2.2)	-0.1 (-1.8, 1.6)	-0.6 (-3.0, 1.9)	*P*=0.662

Mean values (SD); Treatment effect from Generalised Linear Models with baseline value as offset. Adjusted changes and treatment effects are the marginal means adjusted for the covariates age, BMI and gender.

Table B Effects of 10 µg vitamin D_2/d vs. placebo on night-time systolic (SBP) and diastolic (DBP) blood pressure and heart rate in healthy men and women aged 50-70 years.

	Placebo (*n* = 18)	Vitamin D_2 (*n* = 21)	Treatment effect (95% CIs)	P value
SBP, mm Hg				
Baseline 1	106 (14)	107 (10)		
Baseline 2	105 (12)	107 (9)		
Mean baseline	105 (12)	107 (9)		
6 wk	108 (11)	106 (13)		
12 wk	108 (11)	105 (9)		
Mean treatment	108 (11)	105 (10)		
Mean change (95% CIs)	2.7 (0.3, 5.1)	-2.1 (-4.3, 0.1)		
Change adjusted	2.6 (0.4, 4.8)	-2.1 (-4.1, -0.0)	-4.7 (-7.7, -1.7)	*P*=0.002
DBP, mm Hg				
Baseline 1	64 (9)	64 (7)		
Baseline 2	63 (8)	64 (7)		
Mean baseline	63 (8)	64 (6)		
6 wk	65 (7)	64 (8)		
12 wk	65 (8)	63 (7)		
Mean treatment	65 (7)	63 (7)		
Mean change (95% CIs)	1.5 (-0.5, 3.5)	-0.5 (-2.3, 1.4)		
Change adjusted	1.4 (-0.4, 3.2)	-0.4 (-2.0, 1.3)	-1.7 (-4.2, 0.7)	*P*=0.161
Heart rate, beats/min				
Baseline 1	63 (7)	62 (9)		
Baseline 2	63 (8)	64 (10)		
Mean baseline	63 (7)	63 (8)		
6 wk	62 (6)	61 (8)		
12 wk	62 (6)	61 (7)		
Mean treatment	62 (6)	61 (7)		
Mean change (95% CIs)	-1.0 (-3.0, 1.0)	-1.7 (-3.5, 0.1)		
Change adjusted	-1.0 (-2.8, 0.9)	-1.7 (-3., 0.0)	-0.7 (-3.3, 1.9)	*P*=0.592

Mean values (SD); Treatment effect from Generalised Linear Models with baseline value as offset. Adjusted changes and treatment effects are the marginal means adjusted for the covariates age, BMI and gender.

APPENDIX 30 Norms and minimum performance levels for COMPASS tests from the The Brain Performance and Nutrition Research Centre (BPNRC) at Northumbria University.

Test	Norm	Min
Word recognition % Correct	80	55
Word recognition Overall Reaction Time	943	1104
Simple reaction time	289	365
Stroop % Correct	96	78
Stroop Overall Reaction Time	687	919
P&B Average RT Complete	4511	4951
P&B Average RT Thinking	1653	2319
Corsi Block Span Score	6	5